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(21) International Application Number: PCT/US98/01276 (22) International Filing Date: 22 January 1998 (22.01.98) (30) Priority Data: 08/788,973 24 January 1997 (24.01.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/788,973 (CIP) Filed on 24 January 1997 (24.01.97) (71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ELIAS, Peter, M. [US/US]; 210 Stanford Avenue, Mill Valley, CA 94941 (US). BASS, Nathan, M. [US/US]; 3575 22nd Street, San Francisco, CA 94114 (US). HANLEY, Karen [US/US]; 38 Del Casa, Mill Valley, CA 94941 (US). FEINGOLD, Kenneth, R. [US/US]; 214 Forbes, San Rafael, CA 94901 (US).	(74) Agents: HEINES, M., Henry et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: USE OF FXR, PPAR α AND LXR α ACTIVATORS TO RESTORE BARRIER FUNCTION, PROMOTE EPIDERMAL DIFFERENTIATION AND INHIBIT PROLIFERATION (57) Abstract Disorders of the skin and mucous membranes that have a disrupted or dysfunctional epidermal barrier are treated or prevented by topical application of compounds that are either activators of the farnesoid X receptor, activators of the peroxisome proliferator-activated receptor α , and oxysterol activators of the LXR α receptor. The same compounds are also effective in treating disorders of epidermal differentiation and proliferation.		

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5 **USE OF FXR, PPAR α AND LXR α ACTIVATORS**
 TO RESTORE BARRIER FUNCTION,
 PROMOTE EPIDERMAL DIFFERENTIATION
 AND INHIBIT PROLIFERATION

CROSS REFERENCE TO RELATED APPLICATION

10 This application is a continuation-in-part of United States co-pending priority
 application serial no. 08/788,973, filed January 24, 1997 and incorporated herein by
 reference.

GOVERNMENT RIGHTS

15 This invention was made at least in part with assistance from the United States
 Federal Government, under Grant No. HD 29706 of the National Institutes of Health. As
 a result, the Government has certain rights in this invention.

 This invention resides in the technical field of topical formulations for application
 to skin, and to the treatment of subjects suffering from skin or mucous membrane diseases
 or disorders which display disruptions of the barrier function, and those that involve
 disorders of epidermal differentiation and proliferation.

20

BACKGROUND OF THE INVENTION

 One of the functions served by the epidermis in mammals is to form a barrier
 against excessive transcutaneous water loss to the environment. This barrier is formed by
 the anucleate, cornified, outermost layers of the epidermis, collectively known as the

stratum corneum. Localized or generalized perturbations of the epidermal barrier occur in a variety of diseases and conditions of the skin and mucous membrane. These perturbations not only contribute significantly to the morphology of cutaneous lesions, but also activate certain skin diseases such as the Koebner phenomenon in psoriasis and the inflammation in eczematous disorders. The integrity of the barrier is also known to be a major factor in regulating epidermal DNA synthesis. Thus, maintenance of a normal epidermal barrier is a physiological means of inhibiting epidermal hyperproliferation. Examples of conditions that involve or give rise to a disrupted or dysfunctional epidermal barrier are:

- fluid and electrolyte abnormalities, hypothermia, and infection through the skin in premature infants less than 33 weeks of gestational age;
- inflammation to mucous membranes, such as cheilitis, chapped lips, nasal irritation and vulvovaginitis;
- eczematous dermatitides, such as atopic and seborrheic dermatitis, allergic or irritant contact dermatitis, eczema craquelée, photoallergic dermatitis, phototoxic dermatitis, phytophotodermatitis, radiation dermatitis, and stasis dermatitis;
- ulcers and erosions resulting from trauma, burns, bullous disorders, or ischemia of the skin or mucous membranes;
- several forms of ichthyoses;
- epidermolysis bullosae;
- psoriasis;
- hypertrophic scars and keloids;
- cutaneous changes of intrinsic aging and photoaging;
- frictional blistering caused by mechanical shearing of the skin; and
- cutaneous atrophy resulting from the topical use of corticosteroids.

The key constituents of the epidermis that are needed for a functional barrier are the intercellular, lamellar bilayer sheets of stratum corneum lipids. The synthesis of stratum corneum lipids is relatively autonomous from circulating or dietary influences. The synthetic response is regulated instead by alterations in permeability barrier functions. The regulation occurs through changes in the activities, phosphorylation (activation) state, mass, and mRNA for the rate-limiting enzymes of each of the three key lipids: serine palmitoyl transferase (for ceramides), HMGCoA reductase (for cholesterol), and both acetyl CoA carboxylase and fatty acid synthase (for fatty acids). Other results of alterations in barrier function are the regulation of key enzymes of extracellular lipid processing. One such enzyme is β -glucocerebrosidase, which catalyzes the conversion of precursor glycosylceramides into ceramides.

While permeability barrier requirements regulate lipid synthesis, the endogenous regulators of barrier development and homeostasis are not known. Recent studies from the inventors' laboratories have shown that several activators and ligands of the nuclear receptor superfamily, such as glucocorticoids, thyroid hormone, and estrogen, accelerate the appearance of a mature barrier in fetal rodent skin. Hanley, K., *et al.*, "Epidermal barrier ontogenesis: maturation in serum-free media and acceleration by glucocorticoids and thyroid hormone but not selected growth factors," *J. Invest. Dermatol.* 106:404-411 (1996); Hanley, K., *et al.*, "Hormonal basis for the gender difference in epidermal barrier formation in the fetal rat. Acceleration by estrogen and delay by androgen," *J. Invest. Dermatol.* 97:2576-2584 (1996). In contrast, other members of this family, such as 1,25-dihydroxy vitamin D₃, 9-*cis*-retinoic acid, and all-*trans*-retinoic acid, had no effect.

SUMMARY OF THE INVENTION

It has now been discovered that the formation of a mature, fully differentiated stratum corneum and a functional epidermal permeability barrier are accelerated by the topical administration of certain activators of any one of three nuclear receptors -- the farnesoid X-activated receptor (FXR), the peroxisome proliferator-activated receptor α (PPAR α), and the liver-based receptor known as LXR α . These three receptors are nuclear receptors and are part of the nuclear receptor superfamily of transcription factors. The three receptors reside in a subgroup of the superfamily, all receptors in the subgroup sharing the feature that they function only when having formed heterodimers with the retinoid X receptor (RXR). Many other members of the subgroup however do not have activators that accelerate the formation of a mature stratum corneum or barrier development -- these include the vitamin D receptor (VDR), the all-*trans*-retinoic acid receptors (RAR α , β , δ), and the 9-*cis*-retinoic acid (RXR) receptor. The ability of FXR, PPAR α and LXR α activators to achieve this result is therefore unique among members of this subgroup.

The ability of FXR activators to accelerate barrier development is particularly surprising since compounds similar in structure to farnesol (a prominent FXR activator) that are not themselves FXR activators do not accelerate barrier development, despite the similarity in structure to those that do. Also surprising is the ability of the PPAR α receptor, since other PPAR receptors exist (with their own separate activators) that are similar in structure and function, and yet only activators of the PPAR α receptor accelerate barrier development. A further surprising aspect of this discovery is that the barrier development acceleration associated with PPAR α activation is not related to any distinction between essential and non-essential fatty acids, but rather to certain common structural

features. A still further surprising aspect of the discovery relates to oxysterols that are not activators of LXR α but are very close in structure to those that are. The oxysterols that are not LXR α activators do not produce the beneficial results of this invention despite their similarity in structure. Furthermore, many of the activators that are the subject of this invention have never before been known to have any utility as topical epidermal agents.

This newly discovered activity of the three classes of activators renders them useful in the treatment of mammalian skin suffering deficient or perturbed barrier function. The invention is particularly useful in the treatment of premature infants, particularly those less than 33 weeks of gestational age. This invention is also useful for alterations in epidermal differentiation and proliferation. Applications include melanoma and non-melanoma skin cancers and skin precancers, disorders of epidermal differentiation and proliferation such as psoriasis, atopic dermatitis, and various types of ichthyosis with or without an associated barrier abnormality; and benign neoplasms such as warts, condylomata, and seborrheic keratoses.

Other features and advantages of the invention will become apparent from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a bar graph showing trans-epidermal water loss (TEWL) test results of six compounds, two of which are within the scope of this invention.

FIG. 2 is a bar graph showing TEWL test results of five additional compounds, three of which are within the scope of this invention.

FIG. 3 is a bar graph showing TEWL test results of seven additional compounds, one of which is within the scope of this invention.

FIG. 4a is a plot of TEWL vs. concentration of active ingredient for two compounds within the scope of this invention.

FIG. 4b is a plot of TEWL vs. concentration of active ingredient for a third compound within the scope of this invention.

FIG. 4c is a plot of TEWL vs. concentration of active ingredient for a fourth compound and a fifth compound within the scope of this invention.

FIG. 5a is a bar graph showing TEWL test results for two compounds within the scope of this invention at suboptimal levels, both individually and in combination.

FIG. 5b is a bar graph showing TEWL test results for the same two compounds as FIG. 5a except at optimal levels, both individually and in combination.

FIG. 6a is a bar graph showing levels of involucrin mRNA in cell cultures treated with clofibrate. FIG. 6b is a bar graph showing levels of transglutaminase mRNA in cell cultures treated with clofibrate.

FIG. 7a is a further bar graph showing levels of involucrin mRNA in cell cultures treated with clofibrate. FIG. 7b is a further bar graph showing levels of transglutaminase mRNA in cell cultures treated with clofibrate. The calcium ion levels in the media represented by FIGS. 7a and 7b are higher than those of FIGS. 6a and 6b.

FIG. 8 is a bar graph showing levels of involucrin and transglutaminase mRNA in cell cultures treated with clofibrate at different doses.

FIG. 9a is a bar graph showing levels of involucrin protein in cell cultures treated with clofibrate at different doses. Further 9b is a further bar graph showing similar data derived from media containing a higher calcium content.

FIG. 10 is a bar graph showing levels of involucrin protein in cell cultures treated with farnesol at different doses.

FIG. 11 is a bar graph showing levels of involucrin protein in cell cultures treated with juvenile hormone III at different doses.

FIG. 12 is a bar graph showing levels of increase in the rate of cornified envelope formation in cell cultures treated with farnesol and juvenile hormone III at different doses.

FIG. 13 is a bar graph showing the degree of decrease in DNA content in cell cultures treated with farnesol and juvenile hormone III at different doses.

FIG. 14a is a bar graph showing levels of involucrin and transglutaminase mRNA levels in cell cultures treated with two LXR α activators as well as two related compounds (for comparison). FIG. 14b is a bar graph with similar data and a comparison against a further related compound.

FIG. 15 is a bar graph showing the dependency of involucrin and transglutaminase mRNA levels on the dosage of an LXR α activator.

FIG. 16 is a bar graph showing results similar to those of FIGS. 14a and 14b except using a cell culture with a higher calcium content.

FIG. 17a is a bar graph showing involucrin and transglutaminase protein levels in cell cultures treated with two LXR α activators as well as one treated with cholesterol for comparison. FIG. 17b is a bar graph showing the dependency of the transglutaminase protein level on the dosage of one of the LXR α activators at high calcium concentration. FIG. 17c is a bar graph showing the involucrin protein level in cell cultures treated with two LXR α activators and one with cholesterol at high calcium concentration.

FIG. 18a is a bar graph showing cornified envelope formation in low-calcium cell cultures treated with two LXR α activators as well as one treated with cholesterol for comparison. FIG. 18b is a bar graph showing similar results in high-calcium cell cultures.

FIG. 19 is a bar graph showing the rate of DNA synthesis in low-calcium cell cultures treated with two LXR α activators as well as one treated with cholesterol for comparison.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The farnesoid X-activated receptor (FXR), the peroxisome proliferator-activated receptor α (PPAR α), and the receptor LXR α are members of a superfamily of approximately 150 proteins that bind to *cis*-acting elements in the promoters of their target genes and modulate gene expression in response to hormone activators or ligands. For many of these receptors, the activators are known, while for others, termed "orphan receptors," the activators are unknown. Furthermore, some of these receptors bind to their target genes as dimers consisting of two molecules of the same receptor (homodimers), while others bind as dimers consisting of one molecule each of two different receptors (heterodimers). Prominent among the latter are nuclear receptors that require heterodimerization with the retinoid X receptor, as disclosed by Yu, V.C., *et al.*, "RXR β : a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements," *Cell* 67:1251-1266 (1991). Members of this group include the vitamin D receptor, the thyroid hormone receptor (T₃R), the retinoic acid receptor (RAR), the farnesoid X-activated receptor (FXR), the peroxisome proliferator-activated receptors (PPAR), and LXR α .

The farnesoid X-activated receptor (FXR) was first reported by Forman and coworkers, Forman, B.B., "Identification of a nuclear receptor that is activated by farnesol metabolites," *Cell* 81:687-693 (1995). This receptor is a protein having a relative molecular mass (M_r) of approximately 54,000, and is a vertebrate transcription factor regulated by intracellular metabolites. The receptor is activated by certain farnesoids, *i.e.*, farnesol itself and compounds derived from, and/or similar in structure to, farnesol. These farnesoids include farnesol, farnesal, farnesyl acetate, farnesoic acid, geranylgeraniol, and juvenile hormone III. The chemical name for farnesol is 3,7,11-trimethyl-2,6,10-dodecatrienol, and the chemical name for juvenile hormone III is 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester. Farnesoids and metabolites that do not activate the FXR are geraniol, squalene, methoprene, mevalonate, squalene oxide, squalene dioxide, lanosterol, 24,25-epoxycholesterol, pregnenalone, dehydroepiandrosterone, bile acids, and 25-hydroxycholesterol. FXR activators of particular interest are farnesol (denoting *trans,trans*-farnesol hereinafter), farnesal, methyl farnesyl ether, ethyl farnesyl ether, methyl farnesoate, ethyl farnesoate, 7-methyl-9-(3,3-

dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester, and 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid ethyl ester. Preferred among these are farnesol, farnesal, methyl farnesyl ether, methyl farnesoate, and 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester. Particularly preferred are farnesol and 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester (juvenile hormone III).

Peroxisome proliferator-activated receptors (PPAR) are described in a review article by Schoonjans, K., "Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression," *J. Lipid Res.*

37:907-925 (1996). Three subtypes of PPAR have been identified, and these are designated as α , β (or δ), and γ . The α subtype has been cloned from *Xenopus*, humans, mouse and rat; the β (or δ) subtype from *Xenopus*, humans and mouse; and the γ subtype from *Xenopus*, humans and hamster. The PPARs have a modular structure consisting of six functional domains. The one domain that serves as the DNA-binding domain contains about 66 amino acids and is stabilized by two zinc atoms, each binding to four invariant cysteine residues. Included among the activators for PPAR α are fibrates, and fatty acids other than short-chain ($<C_{10}$) fatty acids, long-chain monounsaturated fatty acids, and dicarboxylic acids, particularly dodecanedioic acid. Also included are lower alkyl, preferably methyl, esters of the fibrates and lower alkyl, preferably methyl, esters of the fatty acids. Fibrates include:

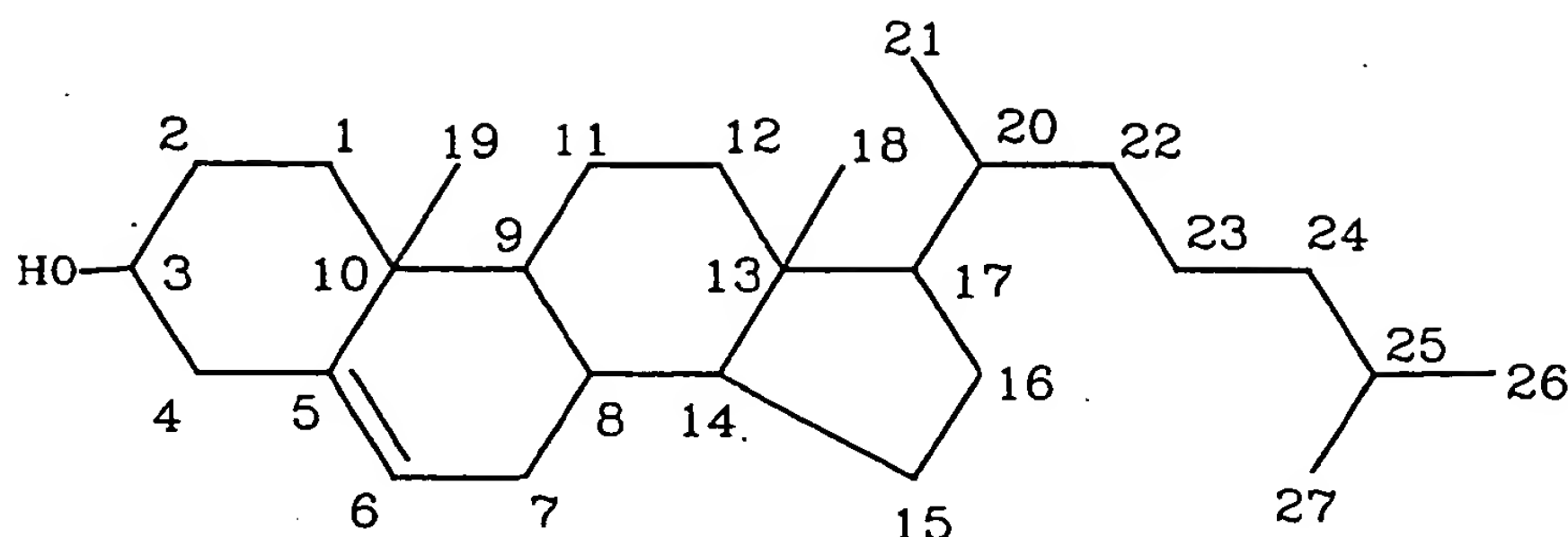
clofibrate: 2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester
 fenofibrate: 2-(4-(4-chlorobenzoyl)phenoxy)-2-methylpropanoic acid isopropyl ester
 ciprofibrate: 2-(4-(2,2-dichlorocyclopropyl)phenoxy)isobutyric acid
 gemfibrozil: 2-(2,4-dimethylphenoxypropyl)-2-methylpropanoic acid
 bezafibrate: 2-(4-(4-chlorobenzoylaminoethyl)phenoxy)-2-methylpropanoic acid

Among the fatty acids, substituted fatty acids are particularly potent activators. PPAR α activators of particular interest are linoleic acid, oleic acid, 5,8,11,14-eicosatetraynoic acid, (4-chloro-6-(2,3-xylidino)-2-pyrimidinyl)thioacetic acid, and clofibrate. A list including these and other examples of PPAR α activators is as follows:

2,4-dichlorophenoxyacetic acid
 2,4,5-trichlorophenoxyacetic acid
 2-methyl-4-chlorophenoxyacetic acid
 2-phenoxy-2-methylpropanoic acid ethyl ester
 2-(4-bromophenoxy)-2-methylpropanoic acid ethyl ester
 2-(4-iodophenoxy)-2-methylpropanoic acid ethyl ester
 2-(2-chlorophenoxy)-2-methylpropanoic acid ethyl ester
 2-(3-chlorophenoxy)-2-methylpropanoic acid ethyl ester
 2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester

- 2-(4-(4-chlorophenyl)phenoxy)-2-methylpropanoic acid ethyl ester
 2-(4-(4-chlorobenzoyl)phenoxy)-2-methylpropanoic acid isopropyl ester
 2-(4-(2,2-dichlorocyclopropyl)phenoxy)-2-methylpropanoic acid
 2-(4-(4-chlorobenzoylaminoethyl)phenoxy)-2-methylpropanoic acid
 5 2-(2,3-dimethyl-4-(1,2,3,4-tetrahydronaphth-1-yl)phenoxy)acetic acid
 2-(2-methyl-3-ethyl-4-(4-chlorobenzyl)phenoxy)acetic acid
 (4-chloro-6-(2,3-xylidino)-2-pyrimidinyl)thioacetic acid
 2-((4-chloro-6-(2,3-xylidino)-2-pyrimidinyl)thioacetamido)ethanol
 perfluoro-*n*-decanoic acid
 10 di-(2-ethylhexyl)adipate
 di-(2-ethylhexyl)phosphate
 di-(2-ethylhexyl)sebacate
 bis-(carboxymethylthio)-1,10-decane
 ethyl 4-(4-chlorophenoxy)butanoate
 15 2-(2-nitro-5-(2-chloro-4-trifluoromethylphenoxy)benzoyloxy)propanoic acid ethyl
 ester
 2-(4-(4-chlorobenzoyl))phenoxy-2-(2-methylpropionamido)ethylsulfonic acid
 tetradecyloxyacetic acid
 tetradecyloxypropionic acid
 20 perfluorobutanoic acid
 perfluorooctanoic acid
 tetradecylthioacetic acid
 tetradecylthiopropionic acid
 di-(2-ethylhexyl)phthalate
 25 mono-(2-ethylhexyl)phthalate
 2-ethylhexanoic acid
 2-propylhexanoic acid

The receptor LXR α was first described by Willy, P.J., *et al.*, "LXR, a nuclear receptor that defines a distinct retinoid response pathway," *Genes & Development* 9:1033-1045 (Cold Spring Harbor Laboratory Press), and is named LXR α due to its initial
 30 isolation from the liver and its liver-rich expression pattern. The activators of LXR α are a subset of oxysterols, including 7 α -hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, 4 β -hydroxycholesterol, 24-hydroxycholesterol, 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, and 20,22-dihydroxycholesterol. Structurally similar compounds
 35 that are not activators of LXR α include cholesterol itself and the oxysterols 7,25-dihydroxycholesterol, 17 α -hydroxycholesterol, and 22(*S*)-hydroxycholesterol (enantiomer of 22(*R*)-hydroxycholesterol). The numbering convention used for substituted
 cholesterol is as follows:



The term "activator" is used in this specification to denote any molecular species that results in activation of the indicated receptor, regardless of whether the species itself binds to the receptor or a metabolite of the species binds to the receptor when the species is administered topically. Thus, the activator can be a ligand of the receptor or it can be an activator that is metabolized to the ligand of the receptor, *i.e.*, a metabolite that is formed in tissue and is the actual ligand.

In the practice of this invention, the activators will be administered as active ingredients in a formulation that is pharmaceutically acceptable for topical administration. These formulations may or may not contain a vehicle, although the use of a vehicle is preferred. Preferred vehicles are non-lipid vehicles, particularly a water-miscible liquid or mixture of liquids. Examples are methanol, ethanol, isopropanol, ethylene glycol, propylene glycol, and butylene glycol, and mixtures of two or more of these compounds.

The concentration of active ingredient in the vehicle will generally range from about 10 μM to about 1000 μM , although for certain active ingredients, the concentration may vary outside this range. In formulations containing farnesol as the active ingredient, preferred concentrations are in the range of about 10 μM to about 100 μM . In formulations containing juvenile hormone III as the active ingredient, preferred concentrations are in the range of about 10 μM to about 200 μM . In formulations containing clofibrate as the active ingredient, preferred concentrations are in the range of about 100 μM to about 1,000 μM . In formulations containing oleic acid as the active ingredient, preferred concentrations are in the range of about 100 μM to about 1000 μM . In formulations containing linoleic acid as the active ingredient, preferred concentrations are in the range of about 5 μM to about 50 μM .

Topical formulations containing the FXR or PPAR α activators in accordance with the present invention are applied to beneficial effect to skin and/or mucus membranes. The activators can be formulated as lotions, solutions, gels, creams, emollient creams, unguents, sprays, or any other form that will permit topical application. The formulation

may also contain one or more agents that promote the spreading of the formulation over the affected area, but are otherwise biologically inactive. Examples of these agents are surfactants, humectants, wetting agents, emulsifiers, or propellants.

5 Amounts that are referred to herein as effective in enhancing barrier development are any amount that will cause a substantial relief of the symptoms of a disrupted or dysfunctional epidermal permeability barrier when applied repeatedly over time. The optimum amounts in any given instance will be readily apparent to those skilled in the art or are capable of determination by routine experimentation.

10 Examples of skin conditions that are susceptible to treatment by the practice of this invention are:

the skin of premature infants of gestational age less than 33 weeks;

atopic and seborrheic dermatitis;

inflammation to mucous membranes, such as cheilitis, chapped lips, nasal irritation and vulvovaginitis;

15 eczematous dermatitis resulting from allergic and irritant contact, eczema craquelée, radiation and stasis dermatitis;

ulcers and erosions due to chemical or thermal burns, bullous disorders, or vascular compromise or ischemia including venous, arterial, embolic or diabetic ulcers;

20 ichthyoses, with or without an associated barrier abnormality;

epidermolysis bullosa;

psoriasis;

hypertrophic scars and keloids;

intrinsic aging and/or dermatoheliosis;

25 mechanical friction blistering;

corticosteroid atrophy; and

melanoma and non-melanoma skin cancer, including lignin melanoma, basal cell carcinoma, squamous cell carcinoma, actinic keratoses, and virally induced neoplasia (warts and condylomata accuminata).

30 Optimal methods and frequency of administration will be readily apparent to those skilled in the art or are capable of determination by routine experimentation. Effective results in most cases are achieved by topical application of a thin layer over the affected area, or the area where one seeks to achieve the desired effect. Depending on the condition being addressed, its stage or degree, and whether application is done for
35 therapeutic or preventive reasons, effective results are achieved with application rates of from one application every two or three days to four or more applications per day.

The invention is generally applicable to the treatment of the skin of terrestrial mammals, including for example humans, domestic pets, and livestock and other farm animals.

5 The following examples are offered for purposes of illustration, and are not intended to limit nor to define the invention. All literature citations in these examples and throughout this specification are incorporated herein by references for all legal purposes to be served thereby.

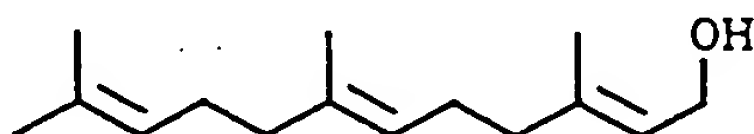
Materials and Methods for Examples 1 Through 11

A. Organ Culture Model and Measurement of Barrier Function

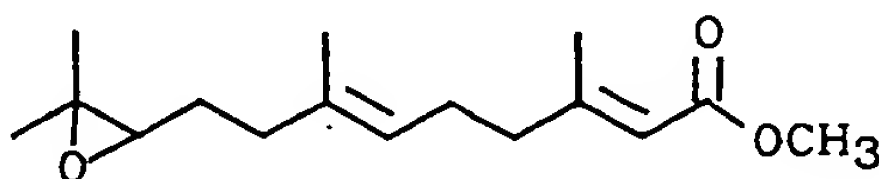
10 Timed pregnant Sprague-Dawley rats (plug date = day 0) were obtained from Simonsen (Gilroy, California, USA) and fetuses were delivered prematurely on day 17. Transepidermal water loss (TEWL) was measured in excised full-thickness flank skin from the fetal rats after various times in culture. The skin explants were placed dermis-side
15 down onto collagen membrane inserts (3μ pore size) in medium M-199 (serum-free), and submerged, and the lateral edges and dermal surface were sealed with petrolatum, such that water loss occurred only through the epidermal surface. Explant samples were weighed hourly, at ambient temperature ($24 \pm 3^\circ\text{C}$) and humidity ($40 \pm 5\%$), over 6 hours using a Cahn balance (sensitivity 0.001 mg). TEWL levels are reported as
20 milligrams of water lost per square millimeter of epidermal surface per hour.

The compounds used in these examples are as follows:

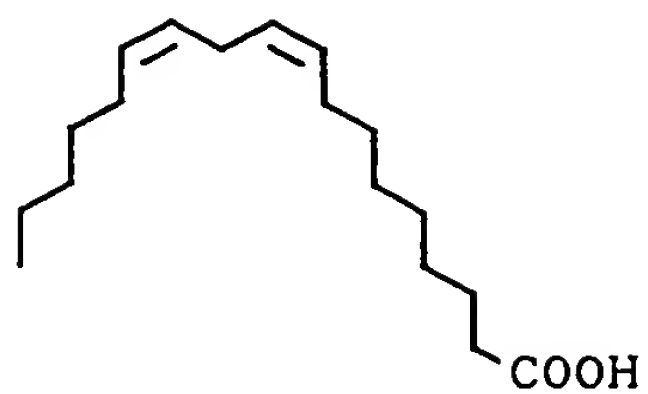
farnesol



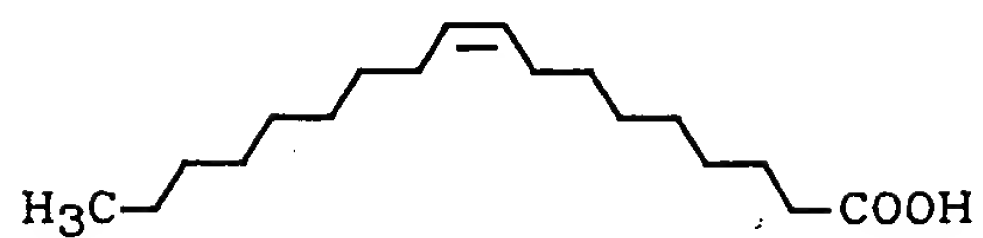
juvenile hormone III



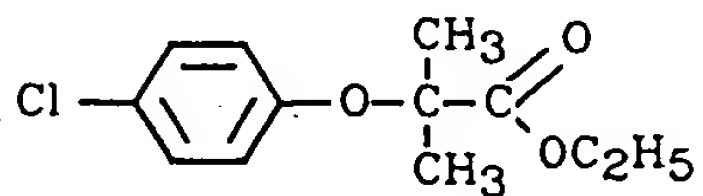
linoleic acid



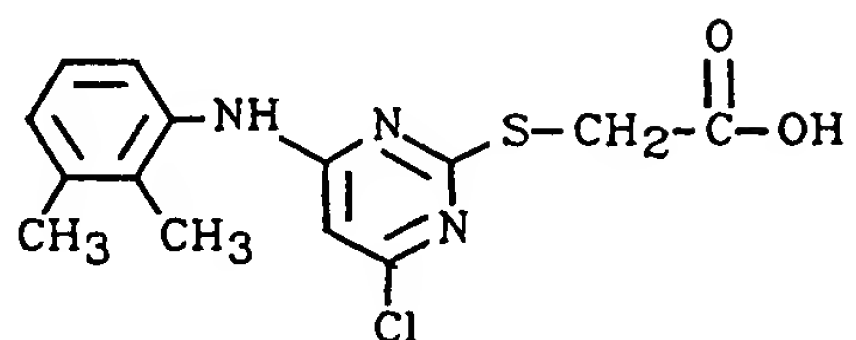
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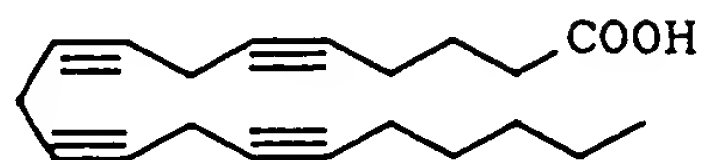
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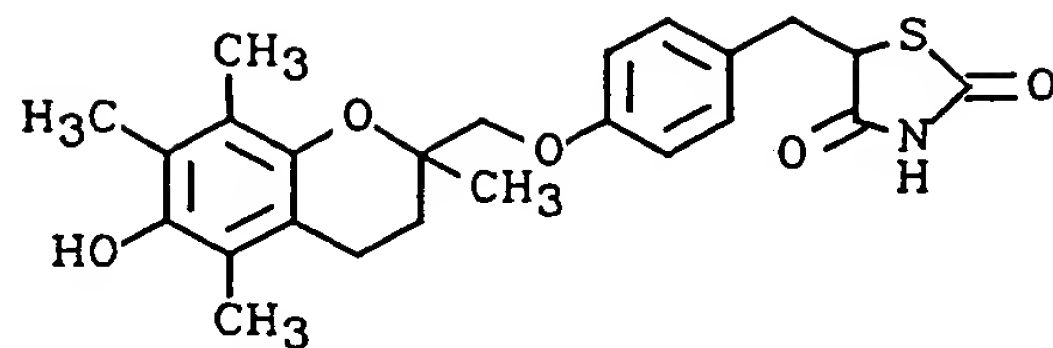
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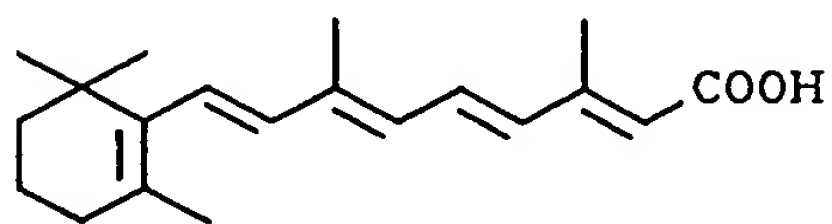
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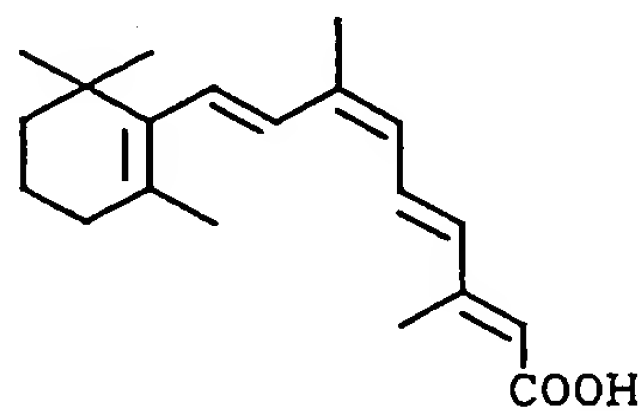
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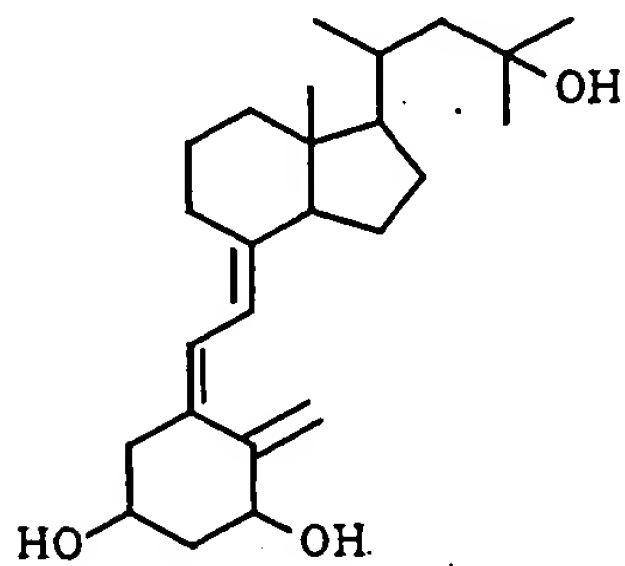
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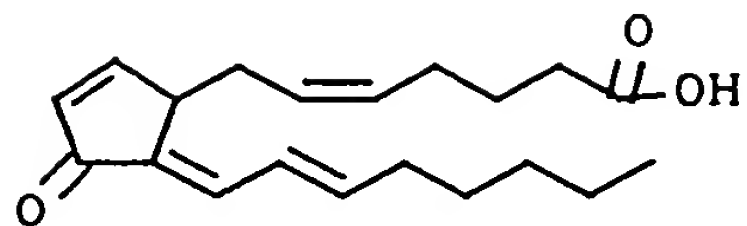
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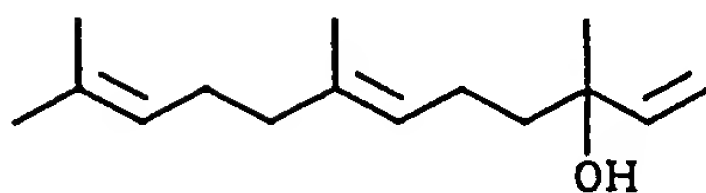
1,25-dihydroxy vitamin D



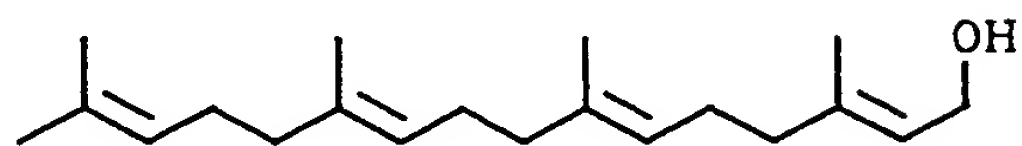
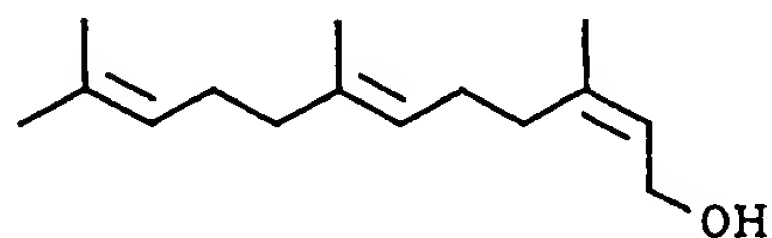
prostaglandin J2



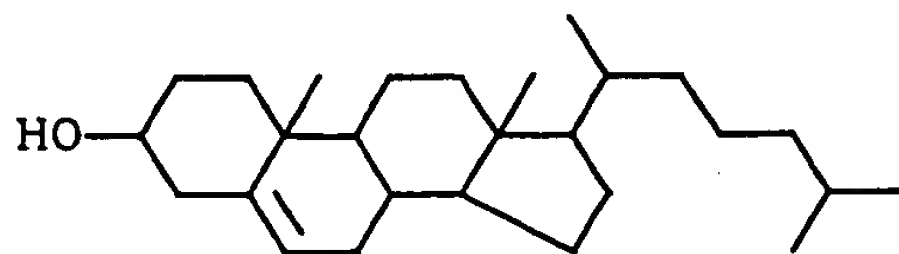
nerolidol



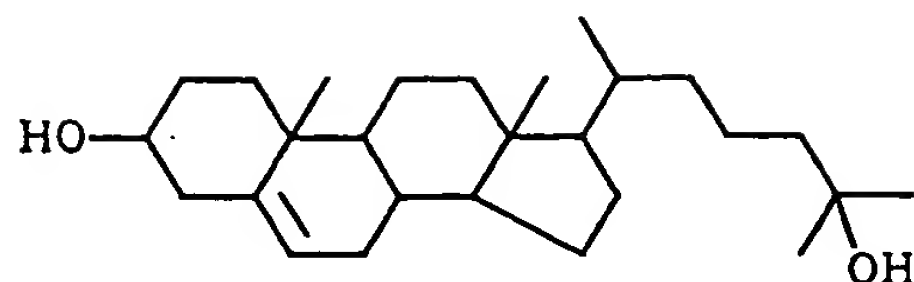
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*cis,trans*-farnesol

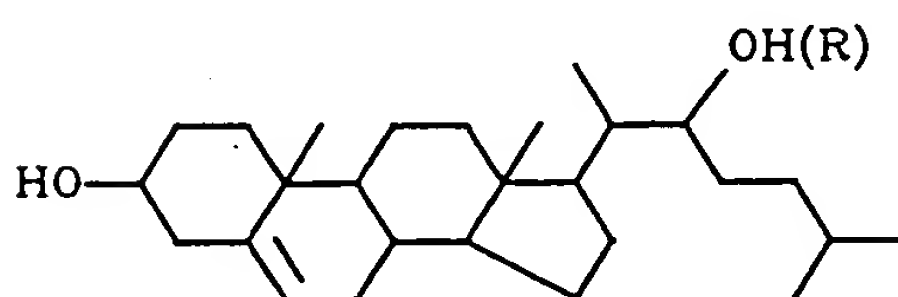
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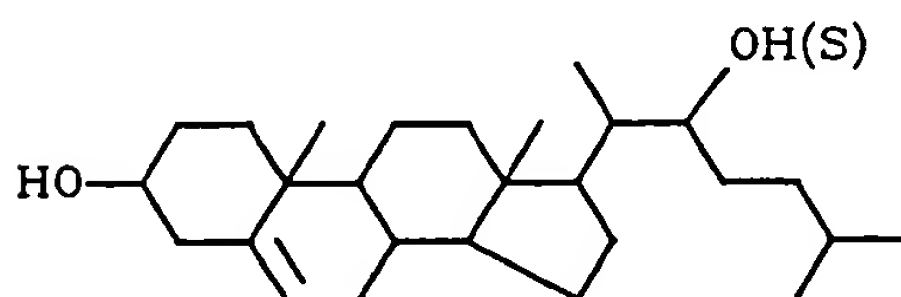
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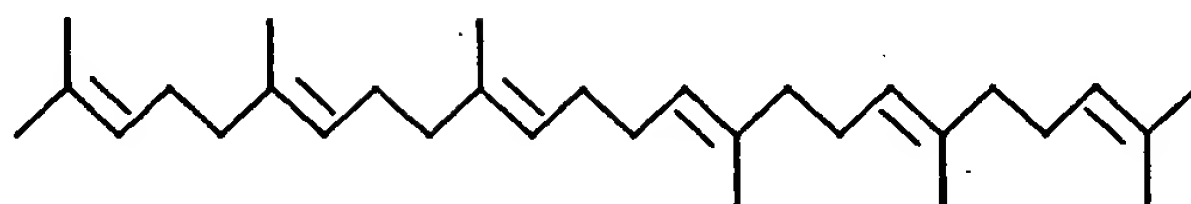
22(R)-hydroxycholesterol



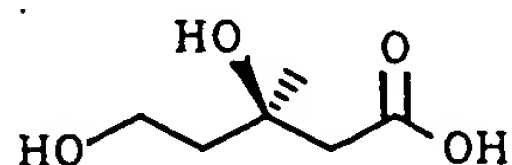
22(S)-hydroxycholesterol



squalene



mevalonate



Prostaglandin J2 was obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA). Troglitazone was obtained from Parke-Davis Laboratories (Detroit, Michigan, USA). The compound (4-chloro-6-(2,3-xylidino)-2-pyrimidinyl)thioacetic acid (known both as pirinixic acid and WY 14,643) were obtained from Wyeth Laboratories,

Philadelphia, Pennsylvania, USA). The compounds *cis*-farnesol, nerolidol, and geranylgeraniol were obtained from University of California, Los Angeles, California, USA. All other compounds were obtained from Sigma Chemical Company (St. Louis, Missouri, USA).

5 Fatty acids and clofibrate (p-chlorophenoxyisobutyric acid) were added to the medium bound to 0.5% (weight/volume) bovine serum albumin (BSA). Isoprenoids and oxysterols were added in ethanol ($\leq 0.1\%$) and juvenile hormone III was added as a dimethylsulfoxide (DMSO) solution ($\leq 0.1\%$). Control explants were incubated in the presence of the appropriate vehicle ($\leq 0.1\%$ DMSO or ethanol, and/or $\leq 0.5\%$ BSA).

10 B. Light and Electron Microscopy

Samples for light microscopy were fixed in modified Karnovsky's solution, plastic-embedded, and $0.5\ \mu\text{m}$ sections were stained with toluidine. Samples for electron microscopy were minced into $1\ \text{mm}^3$ pieces, fixed in modified Karnovsky's fixative, and processed. Sections were stained with uranyl acetate and lead citrate, post-fixed in
15 ruthenium tetroxide, and examined using a Zeiss 10A electron microscope.

C. Tissue Preparation for Enzyme Assays

Epidermis was separated from dermis after incubation in 10 mM ethylenediamine tetraacetic acid (EDTA) in Ca^{++} - and Mg^{++} -free phosphate-buffered saline (PBS), pH 7.4, at 37°C for 30-40 minutes. The tissues were then minced, and homogenized on ice (three
20 times at 15 seconds each with a Polytron homogenizer, followed by sonication twice at ten seconds each at 35% power) in either PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton X-100 (for β -glucocerebrosidase) or in 10 mM Tris (pH 7.5) containing 0.15 M sucrose and 2 mM EDTA (for steroid sulfatase).
 β -Glucocerebrosidase activity was measured in the supernatant following centrifugation at
25 $10,000\times g$ for fifteen minutes at 4°C . Steroid sulfatase activity was measured in the microsomal fraction resulting from a $10,000\times g$ centrifugation (10 minutes, 4°C) followed by 60 minutes of $100,000\times g$ ultracentrifugation at 4°C . Protein content was measured by conventional techniques.

D. β -Glucocerebrosidase and Steroid Sulfatase Activities

β -Glucocerebrosidase activity was assayed using the synthetic substrate 4-methylumbelliferyl β -D-glucoside (4-MUG). The assays were performed in 5 mM sodium taurocholate in citrate-phosphate buffer (pH 5.6) with 0.5 mM 4-MUG for sixty minutes at 37°C, with a final assay volume of 100 μ L, and protein concentration of 1-2 mg/mL. The reaction was terminated with 2 mL of carbonate-bicarbonate buffer (pH 10.5). The fluorescence was then measured at 360 λ (excitation) and 450 λ (emission) and compared with a standard 4-methylumbelliferone (4-MU) curve.

Steroid sulfatase activity was measured by incubating 100 μ g of microsomal protein in 0.1 M Tris containing 5.6 mM glucose (pH 7.4) with 15 μ M (3 H) dihydroepiandrosterone sulfate (DHEAS) (15 μ Ci) in a final volume of 1.1 mL. The product, (3 H) DHEA, was extracted with benzene and an aliquot counted by scintillation spectrophotometry.

E. Statistical Analysis

Statistical evaluation was performed using a Student's test.

EXAMPLE 1

The experiments reported in this example demonstrate that not all activators of RXR heterodimers accelerate barrier development. Activators of three particular receptors -- the retinoid receptor, the vitamin D receptor, and the peroxisome proliferator-activated receptor γ (PPAR γ) -- were used, and the negative results are demonstrated.

Prior studies have shown that full-thickness skin from gestational day 17 rats, after two days in culture, exhibits lamellar bodies in the granular cells and lamellar material secreted in the stratum corneum interstices, but, like day 19 rat skin *in utero*, the epidermis lacks mature lamellar membrane structures and a competent barrier. Hanley, K., *et al.*, "Epidermal barrier ontogenesis: maturation in serum-free media and acceleration by glucocorticoids and thyroid hormone but not selected growth factors," *J. Invest. Dermatol.* 106:404-411 (1996). In contrast, a stratum corneum with barrier function equivalent to that observed in mature epidermis normally forms by day 4 in culture, corresponding to day 21 *in utero*. Hanley *et al.* (1996).

Skin explants from gestational day 17 rats were incubated in the presence of either various activators or vehicle, and transepidermal water loss was measured after two days. The activators and the concentrations at which they were used were as follows:

9-*cis*-retinoic acid: an activator of the retinoid X receptor (RXR); concentration
1 μ M

all-*trans*-retinoic acid: an activator of the retinoic acid receptor (RAR);
concentration 1 μ M

1,25-dihydroxyvitamin D3: an activator of the vitamin D receptor; concentration
1 μ M

prostaglandin J2: an activator of PPAR γ ; concentration 10 μ M

The results in terms of trans-epidermal water loss (TEWL) are shown in bar-graph form as the top four bars in FIG. 1, which when compared with the control (the bottom bar in the Figure) indicate that none of these four activators had any significant effect on reducing the TEWL and hence promoting the barrier development.

In further tests, whose results are not shown in FIG. 1, the first three activators were tested at concentrations ranging from 1 nM to 1 μ M, for their effect on the rate of barrier development. No effect was seen at any concentrations tested within this range.

These data demonstrate that activators of the retinoid receptors, vitamin D receptor and PPAR γ do not accelerate fetal barrier development.

EXAMPLE 2

The experiments reported in this example demonstrate that activators of PPAR α accelerate barrier development, while activators of other PPAR subtypes do not.

Linoleic acid is an example of an activator of PPAR α , and the TEWL value for explants incubated in 300 μ M linoleic acid for two days is shown in FIG. 1. In contrast to the four ineffective activators discussed in Example 1 and the control, all of which are also shown in FIG. 1, linoleic acid markedly decreased the TEWL ($p < 0.005$, $n = 8$).

The three PPAR subtypes presently known are PPAR α , PPAR δ , and PPAR γ . These subtypes are pharmacologically distinct and differentially activated by various agents. Yu, V.C., *et al.*, "RXR β : a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements," *Cell* 67:1251-1266 (1991). To determine whether barrier development acceleration is attributable specifically to PPAR α , a variety of PPAR activators were tested, as follows:

oleic acid: an activator for PPAR α and suspected of also being an activator for PPAR δ , tested at a concentration of 300 μ M

ETYA: an activator for PPAR α and suspected of also being an activator for PPAR δ , tested at a concentration of 100 μ M

WY 14,643: an activator for PPAR α only, tested at a concentration of 100 μ M

clofibrate: an activator for PPAR α only, tested at a concentration of 300 μ M

5 troglitazone: an activator for PPAR γ only, tested at a concentration of 10 μ M

Also relevant to this study is the test result for prostaglandin J2, an activator for PPAR γ only, which is shown in FIG. 1.

The results of the five activators listed above are shown in bar-graph form in FIG. 2, where two controls are included. All activators of PPAR α , including those known to
10 activate only PPAR α demonstrated a significant positive effect, whereas the PPAR γ activator troglitazone (like the PPAR γ activator prostaglandin J2 shown in FIG. 1) had no effect. These results indicate that activators of PPAR α accelerate barrier development while activators that activate only PPAR γ do not accelerate barrier development.

EXAMPLE 3

15 The experiments reported in this example demonstrate that activators of farnesol X-activated receptor (FXR) accelerate barrier development, while other compounds that are either similar in structure to farnesol, metabolites of farnesol, metabolic precursors of farnesol, or other metabolites of metabolic precursors of farnesol, do not accelerate barrier development.

20 Referring again to FIG. 1, farnesol is included in the bar graph, which lists the TEWL value for explants incubated in 50 μ M farnesol for two days. In contrast to the four ineffective activators discussed in Example 1 and the control, farnesol markedly decreased the TEWL ($p < 0.005$, $n = 8$) to a similar degree as did linoleic acid.

To determine whether the effect on barrier development by farnesol is mediated by
25 FXR, several other compounds related to farnesol in the ways stated or by similarly being known to activated FXR were tested for TEWL. Farnesol is produced by a multi-step metabolic synthesis from acetyl coenzyme A, and one of the key intermediates is mevalonate. As part of the pathway, mevalonate is converted in a rate-limiting step to isopentenyl pyrophosphate which through a series of reactions converts to farnesyl
30 pyrophosphate. The latter is converted directly to farnesol but is also capable of following separate pathways toward the synthesis of compounds such as cholesterol, ubiquinone, dolichol, carotenoids, vitamin D, bile acids and steroid hormones. The farnesol pathway in turn leads to farnesoid metabolites such as farnesal, farnesoic acid, methyl farnesoate, and juvenile hormone III.

This series of experiments therefore included tests on mevalonate (tested at 200 μ M), juvenile hormone III (at 100 μ M) and 25-hydroxy cholesterol (at 50 μ M), as well as nerolidol (at 100 μ M), geranylgeraniol (at 50 μ M), *cis*-farnesol (at 50 μ M), and squalene (at 50 μ M), due the similarities in structure between the latter four compounds and farnesol.

The results of these tests are shown in the bar graph of FIG. 3, which indicates that juvenile hormone III at 100 μ M significantly accelerated barrier formation ($p < 0.005$, $n=6$). By contrast, neither mevalonate at 200 μ M, 25-hydroxy cholesterol at 50 μ M, squalene at 50 μ M, geranylgeraniol at 50 μ M, *cis*-farnesol at 50 μ M, nor nerolidol at 100 μ M significantly affected barrier function. These results indicate that the acceleration of barrier development by farnesol and juvenile hormone III is FXR mediated.

EXAMPLE 4

The experiments reported in this example explore the dose responses of the various PPAR α and FXR activators tested in the preceding examples. Tests for TEWL were conducted as described above, using clofibrate, oleic acid, linoleic acid, farnesol and juvenile hormone III, each over a range of concentrations. The results are plotted in FIGS. 4a (clofibrate and oleic acid), 4b (linoleic acid) and 4c (farnesol and juvenile hormone III). For both clofibrate and oleic acid, maximal effects on barrier development occur at a concentration of approximately 500 μ M, and half-maximal effects occur at approximately 250 μ M. For linoleic acid, the maximal effect occurs at approximately 30 μ M and the half-maximal effect at approximately 12.5 μ M. Farnesol demonstrated a maximal effect at approximately 50 μ M and a half-maximal effect at approximately 20 μ M. Juvenile hormone III demonstrated a maximal effect at approximately 250 μ M and a half-maximal effect at approximately 75 μ M.

EXAMPLE 5

A series of experiments was performed to determine whether activation of both RXR and FXR, or both RXR and PPAR α would result in barrier development to a greater degree than activation of FXR or PPAR α alone, either in a synergistic or additive manner.

Explants were incubated in 9-*cis*-retinoic acid in combination first with clofibrate, then with farnesol, then with both, followed by measurements of TEWL. The

9-*cis*-retinoic acid was tested at a concentration of 1 μ M, while the clofibrate and farnesol were both tested at suboptimal concentrations as determined in Example 4 above -- *i.e.*, clofibrate at 100 μ M and farnesol at 10 μ M. No effect on either function or epidermal morphologic maturation was observed by either combination.

5 Experiments were then performed to determine whether the combination of a PPAR α activator and an FXR activator would produce a synergistic result or an additive result. A first set of experiments was performed using suboptimal concentrations of these two activators (clofibrate at 100 μ M and farnesol at 10 μ M). The results are plotted in the bar graph of FIG. 5a, where they are compared with a control (no activators present).

10 The TEWL value for clofibrate, represented by the bar labeled "Clo," is identical to the data point in FIG. 4a at the same concentration, while the TEWL value for farnesol, represented in FIG. 5a by the bar labeled "Farn," is identical to the data point in FIG. 4c at the same concentration. The TEWL value for the combination of the two activators is represented in FIG. 5a by the bar labeled "Clo + Farn," and shows that barrier
15 development was significantly accelerated, indicating an additive effect of clofibrate and farnesol.

 In a second set of experiments, whose results are shown in FIG. 5b, farnesol and clofibrate were used at their maximal concentrations, as determined in FIGS. 4a and 4c. The TEWL value for clofibrate in FIG. 5b is represented by the bar labeled "Clo" and is
20 identical to the value in FIG. 4a at the maximally effective concentration, while the TEWL value for farnesol is represented in FIG. 5b by the bar labeled "Farn" and is identical to the value in FIG. 4c at the maximally effective concentration. The TEWL value for the combination of the two activators at their maximally effective concentrations is represented in FIG. 5b by the bar labeled "Clo + Farn," and shows that barrier development was not
25 further accelerated as compared to the values for the two activators alone.

 The additive effects of clofibrate and farnesol at suboptimal doses suggest a similar activation pathway toward barrier development. This is further suggested by the lack of synergy in combining the same two activators at maximally effective concentrations. The lack of synergy between 9-*cis*-retinoic acid and either clofibrate or farnesol suggests either
30 that the pathway toward barrier development is independent of RXR activation, or that sufficient endogenous RXR activators were present.

EXAMPLE 6

 This example seeks to determine whether a relationship exists at the light microscope and electron microscope levels between the effects of PPAR α and FXR
35 activators and epidermal maturation.

Explants incubated in various media were examined by light microscopy. The incubation media included control medium (containing no activators) and various media individually containing 300 μ M clofibrate, 100 μ M juvenile hormone III, 300 μ M oleic acid, 30 μ M linoleic acid, 50 μ M farnesol, and various concentrations of 9-*cis*-retinoic acid, all-*trans*-retinoic acid, and 1,25-dihydroxy vitamin D3.

The light microscopy of the epidermis in the control explants showed that these explants lacked both a distinct stratum granulosum and a distinct stratum corneum. In contrast, the explants incubated for 48 hours in the clofibrate-containing medium, the juvenile hormone III-containing medium, the oleic acid-containing medium, the linoleic acid-containing medium, and the farnesol-containing medium all had both a multi-layered stratum granulosum and a stratum corneum. The explants incubated in the retinoic acid-containing media (both 9-*cis* and all-*trans*) and those incubated in the 1,25-dihydroxy vitamin D3-containing medium did not exhibit a distinct stratum corneum, and were morphologically indistinguishable from the controls.

Observations were then made of the ultrastructural maturation of the outer epidermis in explants that had been incubated in the various media and then post-fixed in ruthenium tetroxide. Media identical to those of the preceding paragraph were used, excluding the retinoic acid-containing and 1,25-dihydroxy vitamin D3-containing media. Explants treated with all media containing FXR and PPAR α activators showed multiple arrays of mature lamellar membrane unit structures filling the extracellular domains of the stratum corneum. In neither the controls, the vitamin D3, the all-*trans*-retinoic acid, nor the 9-*cis*-retinoic acid cultures, were the extracellular lamellae in the single-layered stratum corneum organized into mature lamellar membrane unit structures.

These results indicate that stimulation of the functional development of the barrier is accompanied by accelerated epidermal stratification and differentiation and the more rapid appearance of mature lamellar unit structures in the stratum corneum.

EXAMPLE 7

This example investigates the effect of PPAR α and FXR activators on the expression of certain enzymes whose activities increases during barrier formation. It is known in the art that epidermal β -glucocerebrosidase activity increases during stratum corneum and barrier development in the rat *in utero* and *in vitro*, that inhibition of this enzyme prevents normal barrier formation, and that this enzyme is required for barrier homeostasis (*in vivo* barrier function in adult). It has also been shown that hormones that accelerate epidermal barrier formation increase β -glucocerebrosidase activity in fetal skin explants. Steroid sulfatase activity is also known to increase during barrier formation, and

is also stimulated by hormones that accelerate barrier formation. β -Glucocerebrosidase and steroid sulfatase were thus selected as enzymes for this study.

Skin explants from 17-day fetal rats were incubated for 24 or 48 hours in media individually containing clofibrate (300 μ M) and juvenile hormone III (100 μ M), and control media. Enzyme activity was measured and the results are presented in Table I as the mean values of five determinations \pm SEM (standard error of the mean). All p values are ≤ 0.005 compared with controls of the same time period.

TABLE I
Enzyme Activity

		Activity	
		β -Glucocerebrosidase (nmol/min/mg)	Steroid Sulfatase (pmol/h/mg)
10	24-Hour Incubation:		
	Vehicle	1.35 \pm 0.22	5.99 \pm 0.80
	Clofibrate	2.97 \pm 0.30	13.90 \pm 1.10
	Juvenile Hormone III	2.89 \pm 0.20	12.55 \pm 1.25
15	48-Hour Incubation:		
	Vehicle	3.02 \pm 0.45	8.75 \pm 0.91
	Clofibrate	5.35 \pm 0.89	22.51 \pm 2.60
	Juvenile Hormone III	5.20 \pm 0.62	18.23 \pm 2.35

The data in this table indicate that β -glucocerebrosidase activity was approximately two-fold higher in treated explants than in controls after both 24 hours and 48 hours, and that steroid sulfatase activity was also increased 1.6 times over controls after 24 hours, and 2.5 times after 48 hours. These data demonstrate that both clofibrate and juvenile hormone III accelerate the developmental increases in activity of two lipid metabolic enzymes associated with the formation of a competent barrier.

EXAMPLE 8

This example illustrates that activators of PPAR α induce differentiation in keratinocytes, the differentiation being part of the process of developing a mature epidermal barrier. Involucrin and transglutaminase (and their mRNA) were measured as indicators of differentiation, these two proteins being components of the ectoskeleton of the corneocytes of cells of the stratum corneum. The PPAR α activator tested was clofibrate.

Human keratinocyte cells were incubated in culture containing calcium at a concentration of 0.03 mM Ca⁺⁺ (a level that is too low to induce differentiation). Also included in the culture medium were clofibrate at 400 μ M or ETYA at 10 μ M and 20 μ M, while separate cell cultures were maintained as controls with neither clofibrate nor ETYA. The production of mRNA for both involucrin and transglutaminase was measured at intervals over a 48-hour time period (0, 6, 12, 24, and 48 hours) by Northern Blot analysis. The results for involucrin are shown in FIG. 6a and those for transglutaminase are shown in FIG. 6b. In each case, the lighter bars represent the control cells and the darker bars the cells incubated in the clofibrate-containing medium. The degree of mRNA generated is expressed as a percent of the control at zero hours. The data in the figures indicates that clofibrate-treated cells exhibit significantly increased levels of both involucrin beginning at six hours and transglutaminase beginning at twelve hours. Similar results were obtained with ETYA.

The experiments were then repeated, except with the calcium at a concentration of 1.2 mM Ca⁺⁺ in the media, a concentration high enough to induce differentiation by itself. The results for involucrin are shown in FIG. 7a and those for transglutaminase are shown in FIG. 7b. In these figures as well, the lighter bars represent the control cells and the darker bars the cells incubated in the clofibrate-containing medium, and the degree of mRNA generated is expressed as a percent of the control at zero hours. In the control cells, the involucrin mRNA level increases to a maximum at 24 hours, then declines by 48 hours, while the transglutaminase mRNA rises for the first 24 hours, then either levels off or continues to increase at a modest rate. The clofibrate-treated cells exhibit increased mRNA levels of both involucrin and transglutaminase at all time points relative to the controls. Similar results were obtained with ETYA.

The dose dependency of clofibrate on the levels of the two mRNAs was determined by a series of incubations for 24 hours using media containing varying concentrations of clofibrate ranging from 0 (vehicle only) to 400 μ M, in either 0.03 mM or 1.2 mM Ca⁺⁺. The results in terms of both involucrin and transglutaminase mRNA levels are shown in FIG. 8, where the degree of mRNA generated is expressed as a percent of the respective measurements taken at zero hours. The mRNA levels for each protein are shown to increase with increasing dosages within this range.

Measurements of the level of the involucrin protein itself were performed in the culture medium by Western Blot. Various incubations were performed, in media containing clofibrate at concentrations ranging from 0 (vehicle only) to 400 μM , and calcium ion at both 0.03 mM and 1.2 mM. The results are shown in FIG. 9a for tests with calcium ion at 0.03 mM, and in FIG. 9b for tests with calcium ion at 1.2 mM. In these figures, the protein contents are expressed as percents of the respective measurements taken from media incubated for the same period of time but in the absence of clofibrate. At both calcium levels, substantial increases in the protein content were observed as the clofibrate concentration was increased within this range. Together, these data demonstrate that PPAR α activators have a profound effect on the expression of protein markers of epidermal differentiation.

EXAMPLE 9

This example demonstrates the increase in keratinocyte differentiation by FXR activators. This is an indication of increased expression of key protein markers of epidermal differentiation. The activators used were farnesol and juvenile hormone III.

The procedures of Example 8 were followed, and measurements were taken on the involucrin protein levels from cells incubated at various concentrations of farnesol and juvenile hormone III ranging from 0 (vehicle only) to 15 μM , in media containing 0.03 mM and 1.2 mM calcium ion, for 24 hours. The results are shown in FIG. 10 for farnesol and FIG. 11 for juvenile hormone III, in 0.03 mM Ca^{++} . The figures show substantial increases in involucrin protein content as the farnesol concentration was increased within this range, and the same was true for juvenile hormone III. These data show that FXR activators increase the expression of key protein markers of epidermal differentiation.

EXAMPLE 10

This example illustrates the effect of clofibrate, farnesol and juvenile hormone III on the rate of cornified envelope formation, as measured by ^{35}S incorporation into detergent- and reducing agent-insoluble protein.

Normal human keratinocytes were cultured to 80% confluence in KGM containing 0.07 μM Ca^{++} . At 80% confluence, the cells were switched to KGM containing 0.03 μM Ca^{++} or 1.2 μM Ca^{++} , plus varying concentrations of clofibrate ranging from 0 to 400 μM . Cells were incubated in these solutions plus ^{35}S -methionine/cysteine for 48

hours. Ionomycin, 5 μ M, was added at 46 hours, two hours before assaying for cornified envelopes. Cells were rinsed with phosphate-buffered saline (PBS) and solubilized in 2% sodium dodecylsulfate, and an aliquot was placed in 4% sodium dodecylsulfate/40 mM dithiothreitol in boiling water for 30 minutes. The sodium dodecylsulfate-insoluble pellet was washed with 0.1% sodium dodecylsulfate/0.1% dithiothreitol, and the radioactivity incorporated into the detergent-insoluble cornified envelope was determined by scintillation counting. To determine total protein synthesized during the 35 S-labeling period, an aliquot of cell lysate before boiling was precipitated with 10% trichloroacetic acid on ice for thirty minutes, washed with 5% trichloroacetic acid, and quantitated by scintillation counting. Percentage of cornified envelope was calculated as percentage cpm/total protein cpm \times 100.

The results are shown in Table II, where they are expressed as percent of the value representing the control with 0.03 mM Ca^{++} , and where each entry represents the mean \pm SEM of two independent experiments. For the data at 0.03 mM Ca^{++} and 200 and 400 μ M clifobrate, and for the data at 1.2 mM Ca^{++} and 400 μ M, the value of p was <0.01 relative to the corresponding vehicle-only controls.

TABLE II
Cornified Envelope Formation Induced by Clofibrate

	Calcium Content in Medium	Clofibrate Content in Medium	Cornified Envelope Formation (% of Control)
20	0.03 mM	0	100.0% \pm 13.9
	0.03 mM	50 μ M	121.4% \pm 15.7
	0.03 mM	200 μ M	321.3% \pm 30.4
	0.03 mM	400 μ M	386.8% \pm 28.7
25	1.2 mM	0	488.0% \pm 34.1
	1.2 mM	50 μ M	458.9% \pm 39.8
	1.2 mM	200 μ M	529.3% \pm 17.4
	1.2 mM	400 μ M	581.9% \pm 28.7

The data in Table II indicate that clofibrate increased cornified envelope formation in both high and low Ca^{++} media.

Similar tests were performed using farnesol at 10 μ M and juvenile hormone III at 15 μ M, individually in separate media at low calcium concentration (0.03 mM). The 48-hour results are shown in FIG. 12, which indicates that both farnesol and juvenile hormone III produced substantial increases in the rate of cornified envelope formation.

EXAMPLE 11

This example illustrates the effect of clofibrate, farnesol and juvenile hormone II on keratinocyte cell growth, demonstrating that both PPAR α and FXR activators inhibit cell growth and proliferation.

- 5 Preconfluent keratinocytes were treated for 48 hours with varying concentrations of clofibrate ranging from 0 (vehicle only) to 400 μ M, in both low (0.03 mM) calcium and high (1.2 mM) calcium. The treated keratinocytes were harvested and sonicated, and the resulting homogenates were incubated with 1 μ L/mL bis-benzimidazole for two hours in the dark. DNA content was quantified by reading the samples on a spectrofluorimeter.
- 10 The results are shown in Table III. Each value in the table is a mean of three samples.

TABLE III
Effect of Clofibrate on Keratinocyte Growth

Clofibrate (μ M)	DNA Content After 48 Hours (μ g/dish)	Significance
0	15.5 \pm 0.9	--
50	14.9 \pm 1.5	not significant
200	12.9 \pm 1.1	p < 0.1
400	11.0 \pm 1.3	p < 0.01

- 15 Similar tests were performed using farnesol at 10 μ M and juvenile hormone III at 15 μ M, both in the presence of 0.03 mM Ca⁺⁺ and with 48 hours of incubation. The results are shown in FIG. 13. Table II and FIG. 13 collectively show that both PPAR α and FXR activators inhibit cell growth.
- 20

Examples 12 through 17 address the activity of oxysterol activators of LXR α in the utilities addressed by this invention.

Materials and Methods for Examples 12 Through 17

A. Cell Culture

Human epidermis was isolated from newborn foreskins and keratinocytes were plated in serum-free keratinocyte growth medium ("KGM"; Clonetics, San Diego, California USA), using conventional techniques. Cells were treated with either the test compounds in vehicle or vehicle alone (<0.1% ethanol) for 24 or 48 hours. The test compounds were obtained from Sigma Chemical Co. (St. Louis, Missouri USA) and were stored as 15 mM stock solutions at -20°C. Mevalonate was solubilized in sterile water.

B. RNA Isolation, Northern Blotting and cDNA Probes

Total RNA was isolated by using TRIZOL (Sigma Chemical Co.), following the manufacturer's protocol. Ethanol-precipitate RNA pellets were suspended in sterile, diethylpyrocarbonate (DEPC)-treated water, and RNA was quantified by absorbance at 260 nm using the 260/280 nm ratio as an index of purity. RNA (15µg per sample) was size fractionated through a 1% agarose gel containing 2.2 M formaldehyde. RNA integrity was visualized following acridine orange staining of the gel following electrophoresis. The RNA was transferred to a nylon membrane that was subsequently baked at 80°C for 2 hours. Blots were hybridized with the appropriate ³²P-labeled probe overnight at 65°C. Washes were then performed in a solution containing 0.1% SSC and 0.1% sodium dodecyl sulfate (SDS) for 20 minutes at room temperature, followed by a 20-minute wash at 65°C. Autoradiography was performed at -70°C. Blots were probed with β-actin to confirm equal loading. Appropriate bands were quantified by densitometry.

C. Involucrin and Transglutaminase Protein Levels

Protein concentration was assessed by protein electrophoresis and Western blotting. Cells were lysed in 2% SDS and the lysate sonicated. Protein determinations were made by use of a Bicinchoninic acid protein assay (Pierce Chemical Company, Rockford, Illinois USA). Following protein determination, equal amounts of protein (50 µg) were separated by electrophoresis on 7.5% polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (0.2-µ, obtained from Bio-Rad Laboratories, Hercules, California USA). Involucrin protein was detected by incubation overnight at 4°C with a polyclonal rabbit anti-human involucrin antibody (1:1000 dilution). The involucrin-specific bands on

the autoradiograms were quantitated by densitometry. Transglutaminase protein expression was measured in a similar manner, using stacking, sample and running buffers containing 4 M urea, and the gels following electrophoresis were washed in 4 M urea, 25 mM TRIS-HCl, pH 7.4, 7.5 mM NaCl, 0.1 mM dithiothreitol (DTT), and 2 mM ethylenediamine tetraacetic acid for 90 minutes prior to electoblotting, allowing the transglutaminase to be detected by antibody. Specific bands on the autoradiograms were quantified by densitometry.

D. Cornified Envelope Formation

To determine the rate of cornified envelope formation, cells were labeled with ^{35}S -methionine/cysteine (2 $\mu\text{Ci/mL}$; trans ^{35}S label, ICN Biomedical, Inc., Irvine, California USA) for 48 hours, incubated for the last 2 hours with 5 μM ionomycin, washed with phosphate-buffered saline (PBS), and harvested into 1.1 mL of 2% SDS. Aliquots were reserved for protein determinations. The remaining cell lysate (1 mL) was sonicated briefly (10 seconds). One mL of 4% SDS/4 mM DTT was then added, and the mixture was heated to $>95^\circ\text{C}$ for 30 minutes. The mixture was then cooled and SDS/DTT-insoluble material was collected on filter discs, washed with 0.5% SDS/0.5% DTT, and quantitated by scintillation spectrophotometry. To determine total protein synthesized during the 48 hours of ^{35}S labeling, a reserved aliquot of the cell lysate (taken prior to heating) was precipitated with an equal volume of 2% bovine serum albumin (BSA) and 1 mL of 10% (weight/volume) trichloroacetic acid (TCA) on ice for thirty minutes, and ^{35}S -labeled precipitated protein was collected onto filters (pore size P8, Fisher Scientific, Pittsburgh, Pennsylvania USA), washed with 5% TCA, and quantified by scintillation spectroscopy. Total protein was determined by conventional methods.

E. DNA Synthesis

The rate of DNA synthesis was determined by measuring the incorporation of ^3H -thymidine into cellular DNA after 16 hours of incubation with 2 μCi ^3H -thymidine per mL of media (110 Ci/mmol methyl-1',2'- ^3H -thymidine (Amersham Laboratories, Arlington Heights, Illinois USA). The cells were then solubilized in 1N NaOH, and the radioactivity in the washed TCA precipitate was quantitated by scintillation spectroscopy.

F. Transfections

Keratinocytes were transfected by passing primary keratinocytes onto 6-well multiwell plates 1-2 days prior to transfection to yield a confluence of 20-40% on the day of transfection. Involucrin promoter construct (1 μ g), 0.1 μ g RSV- β -gal, and 7.5 μ g of polybrene (dihexabromide, Aldrich Chemical Company, inc., Milwaukee, Wisconsin USA) were added in media (KGM containing 0.03 mM Ca^{++}) in a final volume of 0.35 mL, and keratinocytes were incubated at 37°C for 5 hours with gentle shaking each hour. Cells were then rinsed with CMF PBS, followed by incubation at room temperature for three minutes with 10% glycerol in media. Following two rinses with CMF PBS, keratinocytes were incubated overnight with 2 mL of KGM containing 0.03 mM Ca^{++} . Keratinocytes were treated the following day with fresh media (either 0.03 or 1.2 mM Ca^{++}) containing either 10 μ M of the test compound or vehicle (ethanol). Cells were rinsed and harvested in 250 μ L cell lysis buffer. The lysate was spun at 10,000 \times g (4°C) for two minutes, and 20 μ L of supernatant was assayed with luciferase substrate and β -galactosidase substrate. β -Galactosidase activity was used to normalize data and correct for any transfection inefficiencies.

EXAMPLE 12

This example demonstrates that the oxysterol activators of LXR α stimulate keratinocyte differentiation, as indicated by the levels of involucrin and transglutaminase mRNA, while cholesterol, mevalonate, and 22(*S*)-hydroxycholesterol demonstrated no significant effects.

Northern blot analyses were performed on keratinocytes maintained in low calcium (0.03 mM) and incubated for 24 hours in the presence of vehicle alone (<0.1% ethanol) or vehicle containing 25-hydroxycholesterol (10 μ M), 22(*R*)-hydroxycholesterol (10 μ M), cholesterol (10 μ M), or mevalonate (500 μ M) (individually). The mRNA levels of involucrin ("INV") and transglutaminase ("TG'ase") are shown in the bar graph of FIG. 14a, the INV indicated by clear bars and the TG'ase by shaded bars, both with error limits shown. The figure shows that both 25-hydroxycholesterol ("25-OH") and 22(*R*)-hydroxycholesterol ("22R-OH") exhibited an approximate twofold increase in mRNA levels compared to the vehicle alone. In contrast, neither cholesterol ("chol") nor mevalonate ("mev") had any effect.

As a further comparison, 22(*R*)-hydroxycholesterol and 22(*S*)-hydroxycholesterol ("22S-OH") were tested under the same conditions, and the results are shown in the bar graph of FIG. 14b, using the same bar indications as those in FIG. 14a. No effect on

either INV or TG'ase mRNA was observed in the 22S-OH data, while the 22R-OH data repeated what was observed in FIG. 14a.

The dose dependency of 22(*R*)-hydroxycholesterol was then determined, using doses of 0, 0.1 μ M, 1.0 μ M, 10.0 μ M, and 15.0 μ M. The results are shown in the bar graph of FIG. 15, using the same bar indications for INV and TG'ase mRNA as in FIGS. 14a and 14b. The plot indicates that maximal effects were seen with a dosage of 10-15 μ M, and half-maximal effects with approximately 5 μ M.

EXAMPLE 13

An extracellular calcium concentration of 1.2 mM is well known to stimulate differentiation in keratinocytes. This example demonstrates that oxysterol activators of LXR α in the presence of a high calcium concentration stimulate keratinocyte differentiation even further.

The experiment of Example 12 was repeated except that the calcium concentration was raised to 1.2 mM and only the vehicle alone and the vehicle plus 25-hydroxycholesterol (10 μ M), 22(*R*)-hydroxycholesterol (10 μ M), or cholesterol (10 μ M) were tested. The results are shown in the bar graph of FIG. 16, using the same bar indications as in the preceding figures. The bar graph of FIG. 16 shows that 25-hydroxycholesterol and 22(*R*)-hydroxycholesterol both induced mRNA levels of both INV and TG'ase, INV by approximately 2.3-2.4 times the control, and TG'ase by approximately 2.5-2.9 times the control, while cholesterol had no effect on either. Determinations were also made of β -actin mRNA levels, and the results (not shown in the Figures) indicated that these levels were unaffected by oxysterol treatment in either low (0.03 mM) or high (1.2 mM) calcium conditions.

EXAMPLE 14

This example demonstrates that the oxysterol activators of LXR stimulate protein levels of involucrin and transglutaminase, while cholesterol does not.

Levels of involucrin and transglutaminase protein were measured in keratinocytes incubated in low calcium (0.03 mM) and treated with the test compounds for 24 hours. The results are shown in the bar graph of FIG. 17a, where the test compounds are compared with the vehicle alone (control) and cholesterol, using the same bar indications as in FIGS. 14 through 16. The plot shows that 25-hydroxycholesterol induced INV levels approximately 1.7-fold and TG'ase protein levels approximately 1.6-fold relative to the

control, and that 22(*R*)-hydroxycholesterol induced INV levels approximately 3.2-fold and TG'ase protein levels approximately 2.8-fold relative to the control. Cholesterol had no effect.

5 Tests performed in high calcium conditions (1.2 mM) are shown in FIGS. 17b (for 22(*R*)-hydroxycholesterol only, at three dose levels) and 17c (for 22(*R*)-hydroxycholesterol, 25-hydroxycholesterol, and cholesterol, all at a dose of 15 μ M). At a concentration as low as 5 μ M, 22(*R*)-hydroxycholesterol showed a 2.1-fold increase in TG'ase protein level (FIG. 17b). For INV protein, the increase at 15 μ M concentration was 2.8-fold for 22(*R*)-hydroxycholesterol and 3.2-fold for 25-hydroxycholesterol, with essentially no
10 increase for cholesterol (FIG. 17c).

EXAMPLE 15

An additional measure of keratinocyte differentiation is the rate of cornified envelope formation. Using the procedures set forth in the Materials and Methods section above, 25-hydroxycholesterol (10 μ M) and 22(*R*)-hydroxycholesterol (10 μ M) were
15 compared with cholesterol (10 μ M) and the vehicle alone (control), with treatment for 48 hours under both low calcium (0.03 mM) and high calcium (1.2 mM) conditions. The results are shown in the bar graphs of FIGS. 18a (low calcium) and 18b (high calcium). The low calcium results (FIG. 18a) showed that treatment with 25-hydroxycholesterol yielded a 1.6-fold increase in cornified envelope (CE) formation, and treatment with
20 22(*R*)-hydroxycholesterol yielded a 1.3-fold increase. The high calcium results (FIG. 18b) showed that treatment with 25-hydroxycholesterol yielded a 1.75-fold increase in CE formation, and treatment with 22(*R*)-hydroxycholesterol yielded a 2.1-fold increase. Cholesterol itself produced no increase in either low or high calcium conditions.

EXAMPLE 16

25 This example demonstrates that oxysterol activators of LXR α inhibit proliferation of keratinocytes, as indicated by the rate of DNA synthesis in keratinocytes. Oxysterols are known to be potent inhibitors of cell growth in other cell types such as thymocytes and lymphocytes.

Using the procedures set forth in the Materials and Methods section above,
30 25-hydroxycholesterol (10 μ M) and 22(*R*)-hydroxycholesterol (10 μ M) were compared with cholesterol (10 μ M) and the vehicle alone (control), with treatment for 24 hours under low calcium (0.03 mM) condition. The results are shown in the bar graph of FIG. 19. The

bar graph shows that 25-hydroxycholesterol decreased the rate of DNA synthesis to 50% of the control, and 22(*R*)-hydroxycholesterol reduced the rate to 42% of control, during the 16-hour time period in which the DNA synthesis was measured. In contrast, cholesterol modestly but significantly increased DNA synthesis (117% of control).

5

EXAMPLE 17

This example explores whether the increase that oxysterols cause in INV and TG'ase mRNA levels is related to the fact that these oxysterols inhibit the enzyme HMG CoA reductase, which is the rate-limiting enzyme of cholesterol synthesis and which leads to decreased levels of isoprenoids. To answer this question, tests similar to those described in Example 12 above were performed in the presence and absence of mevalonate, which is the earliest product of HMG CoA reductase.

The tests measured INV and TG'ase mRNA levels in keratinocytes treated with 25-hydroxycholesterol (10 μ M), alone or in the presence of mevalonate (10 μ M), as well as mevalonate alone (10 μ M), for 24 hours. Treatment with mevalonate alone had no effect on the INV and TG'ase mRNA levels. Treatment with 25-hydroxycholesterol, both alone and in the presence of mevalonate, resulted in a two-fold increase in the INV and TG'ase mRNA levels. The conclusion is that inhibition of HMG CoA reductase is not the basis for the increase in INV and TG'ase mRNA levels caused by oxysterols.

The foregoing is offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that the concentrations, operating conditions, materials, procedural steps and other parameters of protocols described herein may be further modified or substituted in various ways without departing from the spirit and scope of the invention.

WE CLAIM:

1 1. A method for treating the epidermis of a terrestrial mammalian subject
2 suffering from a condition characterized by a perturbed epidermal barrier function, said
3 method comprising topically administering to said epidermis a topical composition
4 containing an active ingredient that is a member selected from the group consisting of
5 activators of farnesoid X-activated receptor, activators of peroxisome proliferator-activated
6 receptor α , and oxysterol activators of LXR α , said active ingredient being present in a
7 concentration that is effective in enhancing barrier development.

1 2. A method in accordance with claim 1 in which said active ingredient is an
2 activator of farnesoid X receptor.

1 3. A method in accordance with claim 2 in which said activator is a member
2 selected from the group consisting of farnesol, farnesal, methyl farnesyl ether, ethyl
3 farnesyl ether, methyl farnesoate, ethyl farnesoate, 7-methyl-9-(3,3-dimethyloxiranyl)-3-
4 methyl-2,6-nonadienoic acid methyl ester, and 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-
5 2,6-nonadienoic acid ethyl ester.

1 4. A method in accordance with claim 2 in which said activator is a member
2 selected from the group consisting of farnesol, farnesal, methyl farnesyl ether, methyl
3 farnesoate, and 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl
4 ester.

1 5. A method in accordance with claim 2 in which said activator is farnesol.

1 6. A method in accordance with claim 2 in which said activator is 7-methyl-
2 9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester.

1 7. A method in accordance with claim 1 in which said active ingredient is an
2 activator of peroxisome proliferator-activated α receptor.

1 8. A method in accordance with claim 7 in which said activator is a member
2 selected from the group consisting of linoleic acid, oleic acid, 5,8,11,14-eicosatetraynoic
3 acid, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, and clofibrate.

1 9. A method in accordance with claim 7 in which said activator is linoleic
2 acid.

1 10. A method in accordance with claim 7 in which said activator is clofibrate.

1 11. A method in accordance with claim 1 in which said active ingredient is an
2 activator of LXR α receptor.

1 12. A method in accordance with claim 11 in which said activator of LXR α
2 receptor is a member selected from the group consisting of 22(*R*)-hydroxycholesterol, 25-
3 hydroxycholesterol, 7 α -hydroxycholesterol, 24-hydroxycholesterol, 27-hydroxycholesterol,
4 4 β -hydroxycholesterol, 20,22-dihydroxycholesterol, and 20(*S*)-hydroxycholesterol.

1 13. A method in accordance with claim 11 in which said activator of LXR α
2 receptor is 22(*R*)-hydroxycholesterol.

1 14. A method in accordance with claim 11 in which said activator of LXR α
2 receptor is 25-hydroxycholesterol.

1 15. A method in accordance with claim 1 in which the concentration of said
2 active ingredient is from about 10 μ M to about 1000 μ M.

1 16. A method in accordance with claim 1 in which said active ingredient is
2 farnesol and the concentration of said active ingredient is from about 10 μ M to about
3 100 μ M.

1 17. A method in accordance with claim 1 in which said active ingredient is 7-
2 methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester and the
3 concentration of said active ingredient is from about 10 μ M to about 200 μ M.

1 18. A method in accordance with claim 1 in which said active ingredient is
2 clofibrate and the concentration of said active ingredient is from about 100 μ M to about
3 1,000 μ M.

1 19. A method in accordance with claim 1 in which said active ingredient is
2 oleic acid and the concentration of said active ingredient is from about 100 μ M to about
3 1000 μ M.

1 20. A method in accordance with claim 1 in which said active ingredient is
2 linoleic acid and the concentration of said active ingredient is from about 5 μ M to about
3 50 μ M.

1 21. A method for treating the epidermis or mucous membrane of a terrestrial
2 mammalian subject suffering from a condition of disturbed differentiation or excess
3 proliferation, said method comprising topically administering to said epidermis or mucous
4 membrane a topical composition containing an active ingredient that is a member selected
5 from the group consisting of activators of farnesoid X-activated receptor, activators of
6 peroxisome proliferator-activated receptor α , and oxysterol activators of LXR α , said active
7 ingredient being present in a concentration that is effective in normalizing said condition.

1 22. A method in accordance with claim 21 in which said active ingredient is an
2 activator of farnesoid X receptor.

1 23. A method in accordance with claim 22 in which said activator is a member
2 selected from the group consisting of farnesol, farnesal, methyl farnesyl ether, ethyl
3 farnesyl ether, methyl farnesoate, ethyl farnesoate, 7-methyl-9-(3,3-dimethyloxiranyl)-3-
4 methyl-2,6-nonadienoic acid methyl ester, and 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-
5 2,6-nonadienoic acid ethyl ester.

1 24. A method in accordance with claim 22 in which said activator is a member
2 selected from the group consisting of farnesol, farnesal, methyl farnesyl ether, methyl
3 farnesoate, and 7-methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl
4 ester.

1 25. A method in accordance with claim 22 in which said activator is farnesol.

1 26. A method in accordance with claim 22 in which said activator is 7-methyl-
2 9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester.

1 27. A method in accordance with claim 21 in which said active ingredient is an
2 activator of peroxisome proliferator-activated α receptor.

1 28. A method in accordance with claim 27 in which said activator is a member
2 selected from the group consisting of linoleic acid, oleic acid, 5,8,11,14-eicosatetraenoic
3 acid, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, and clofibrate.

1 29. A method in accordance with claim 27 in which said activator is linoleic
2 acid.

3 30. A method in accordance with claim 1 in which said active ingredient is an
4 activator of LXR α receptor.

1 31. A method in accordance with claim 30 in which said activator of LXR α
2 receptor is a member selected from the group consisting of 22(*R*)-hydroxycholesterol, 25-
3 hydroxycholesterol, 7 α -hydroxycholesterol, 24-hydroxycholesterol, 27-hydroxycholesterol,
4 4 β -hydroxycholesterol, 20,22-dihydroxycholesterol, and 20(*S*)-hydroxycholesterol.

1 32. A method in accordance with claim 30 in which said activator of LXR α
2 receptor is 22(*R*)-hydroxycholesterol.

1 33. A method in accordance with claim 27 in which said activator is clofibrate.

1 34. A method in accordance with claim 21 in which the concentration of said
2 active ingredient is from about 10 μ M to about 1000 μ M.

1 35. A method in accordance with claim 21 in which said active ingredient is
2 farnesol and the concentration of said active ingredient is from about 10 μ M to about
3 100 μ M.

1 36. A method in accordance with claim 21 in which said active ingredient is 7-
2 methyl-9-(3,3-dimethyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester and the
3 concentration of said active ingredient is from about 10 μ M to about 200 μ M.

1 37. A method in accordance with claim 21 in which said active ingredient is
2 clofibrate and the concentration of said active ingredient is from about 100 μ M to about
3 1,000 μ M.

1 38. A method in accordance with claim 21 in which said active ingredient is
2 oleic acid and the concentration of said active ingredient is from about 100 μ M to about
3 1000 μ M.

1 39. A method in accordance with claim 21 in which said active ingredient is
2 linoleic acid and the concentration of said active ingredient is from about 5 μ M to about
3 50 μ M.

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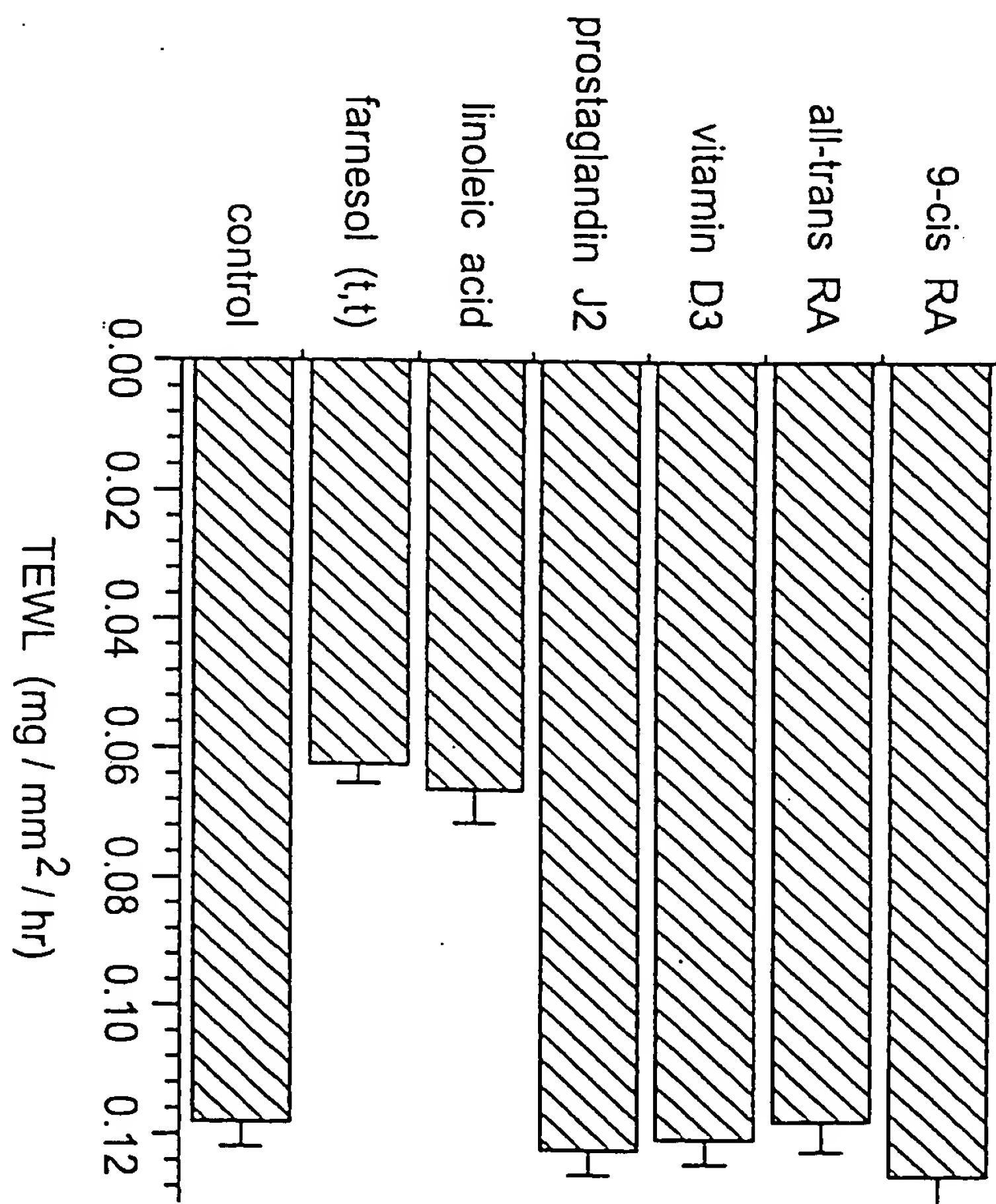


Fig. 1

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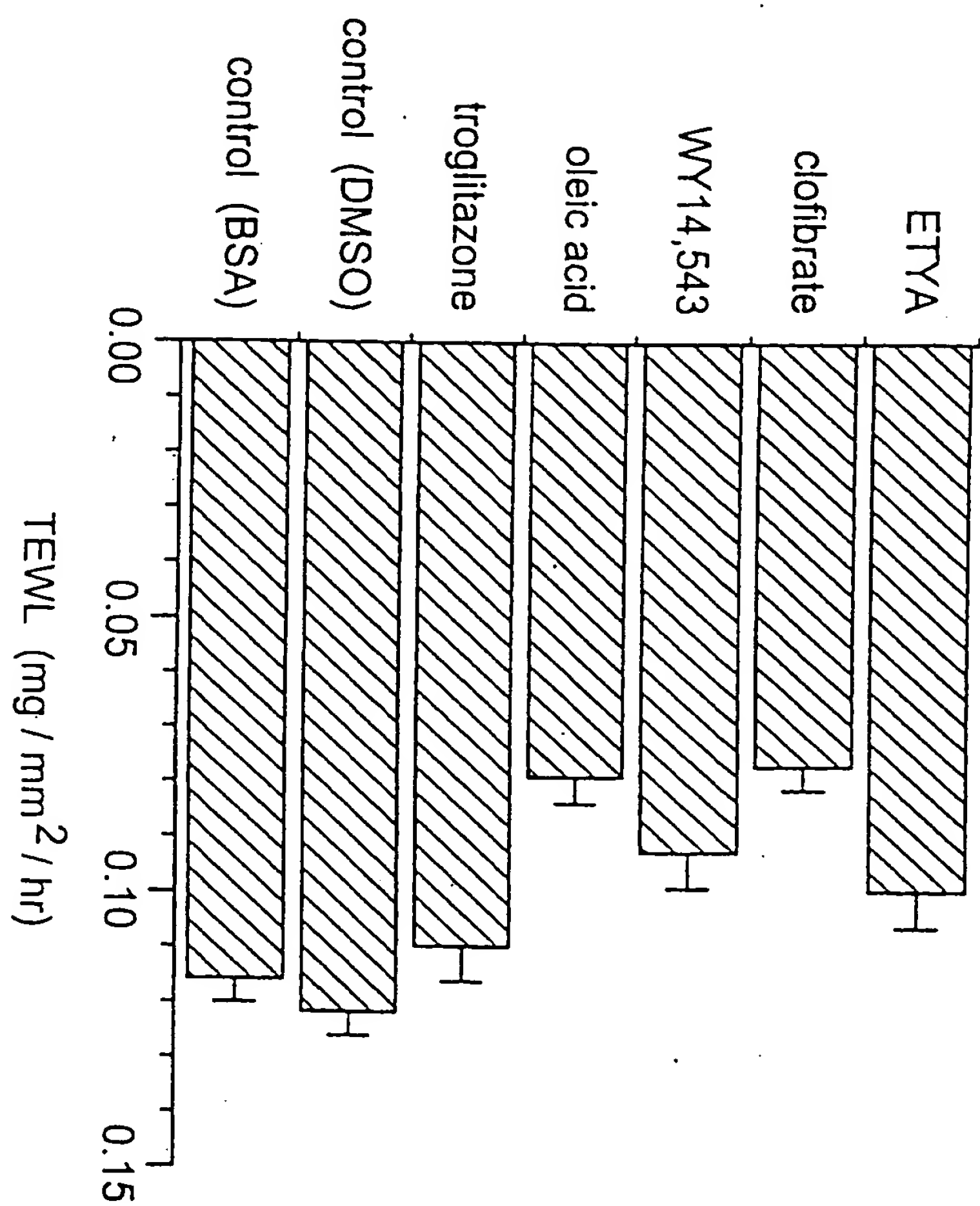


Fig. 2

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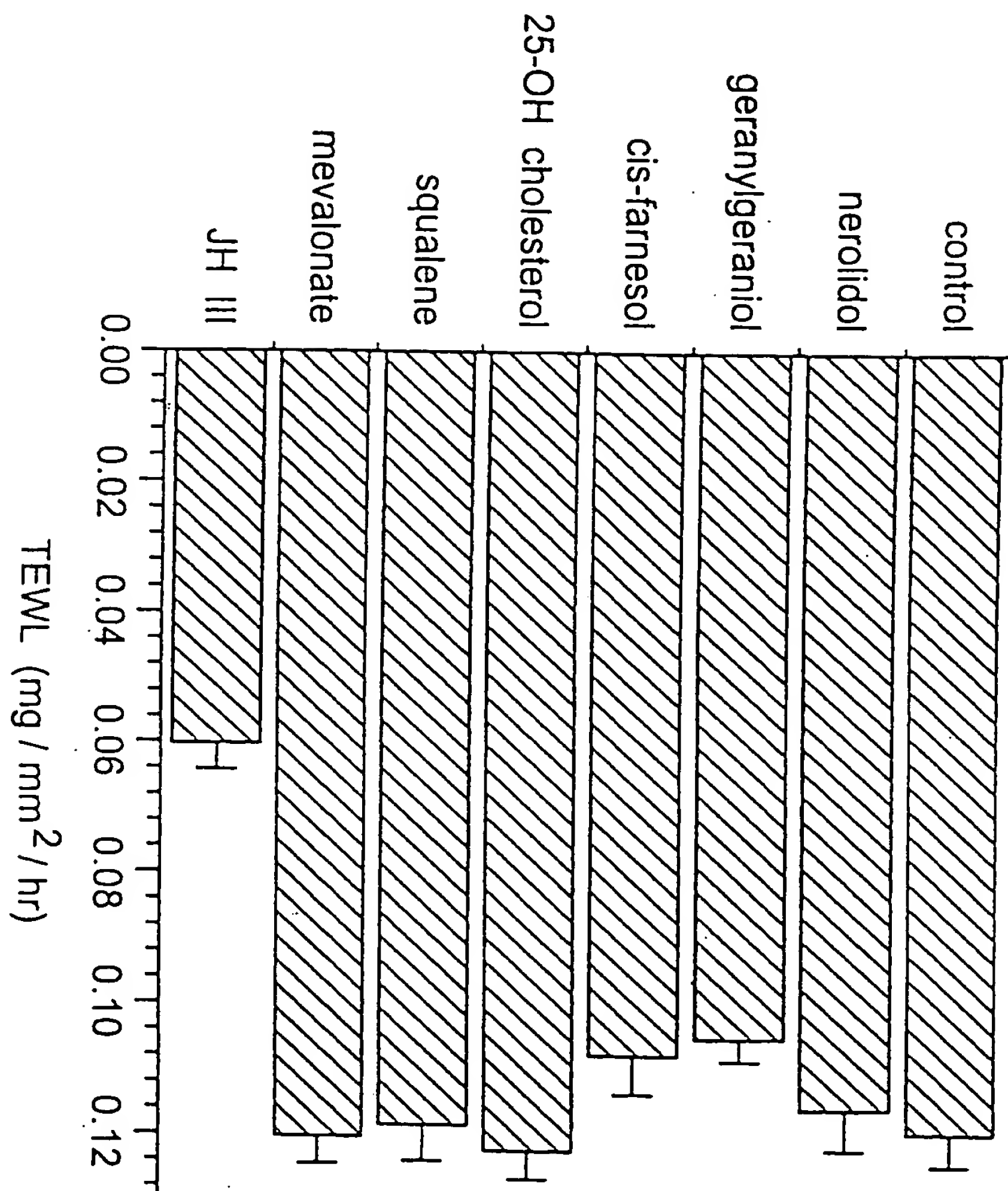


Fig. 3

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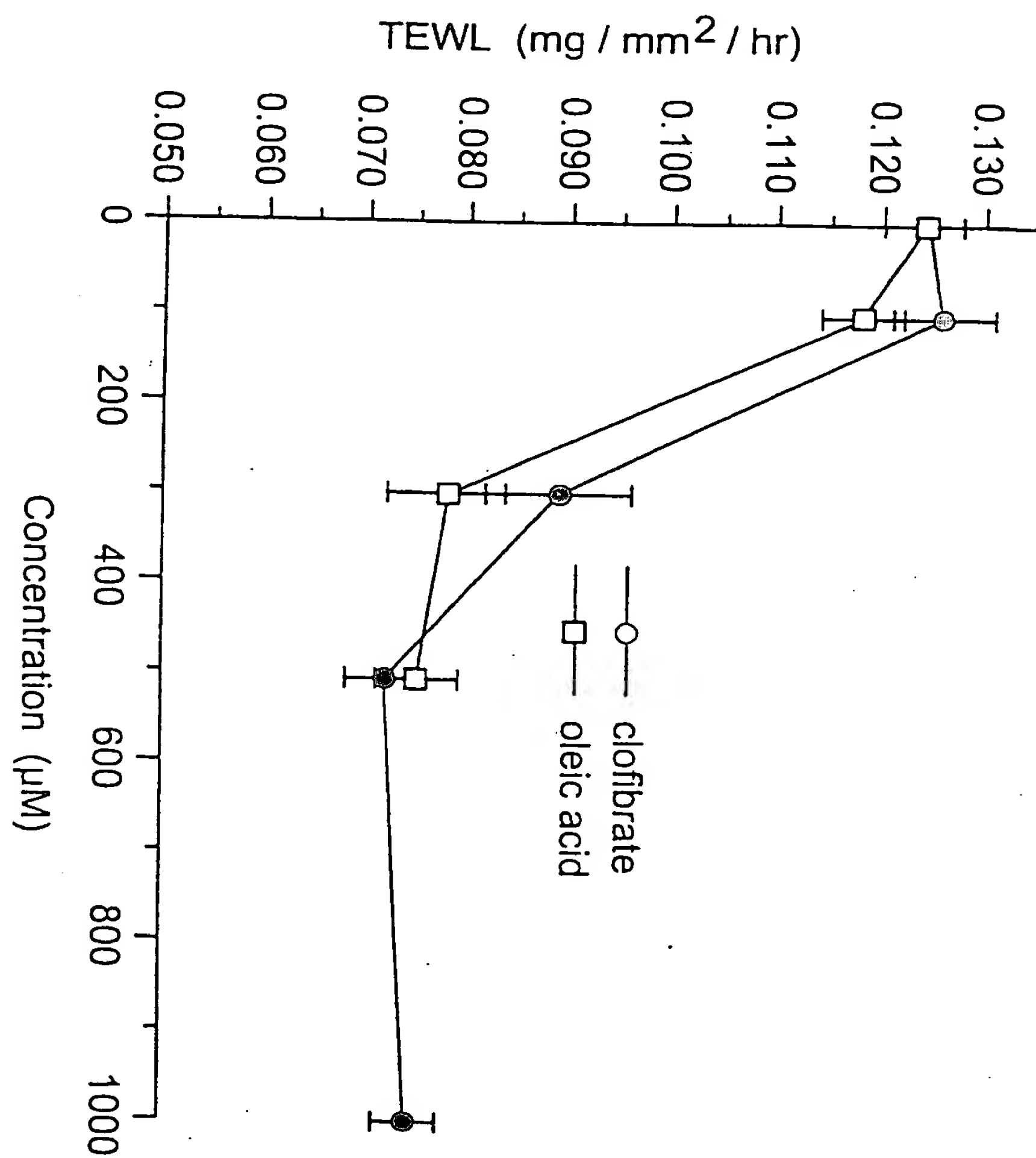


Fig. 4a

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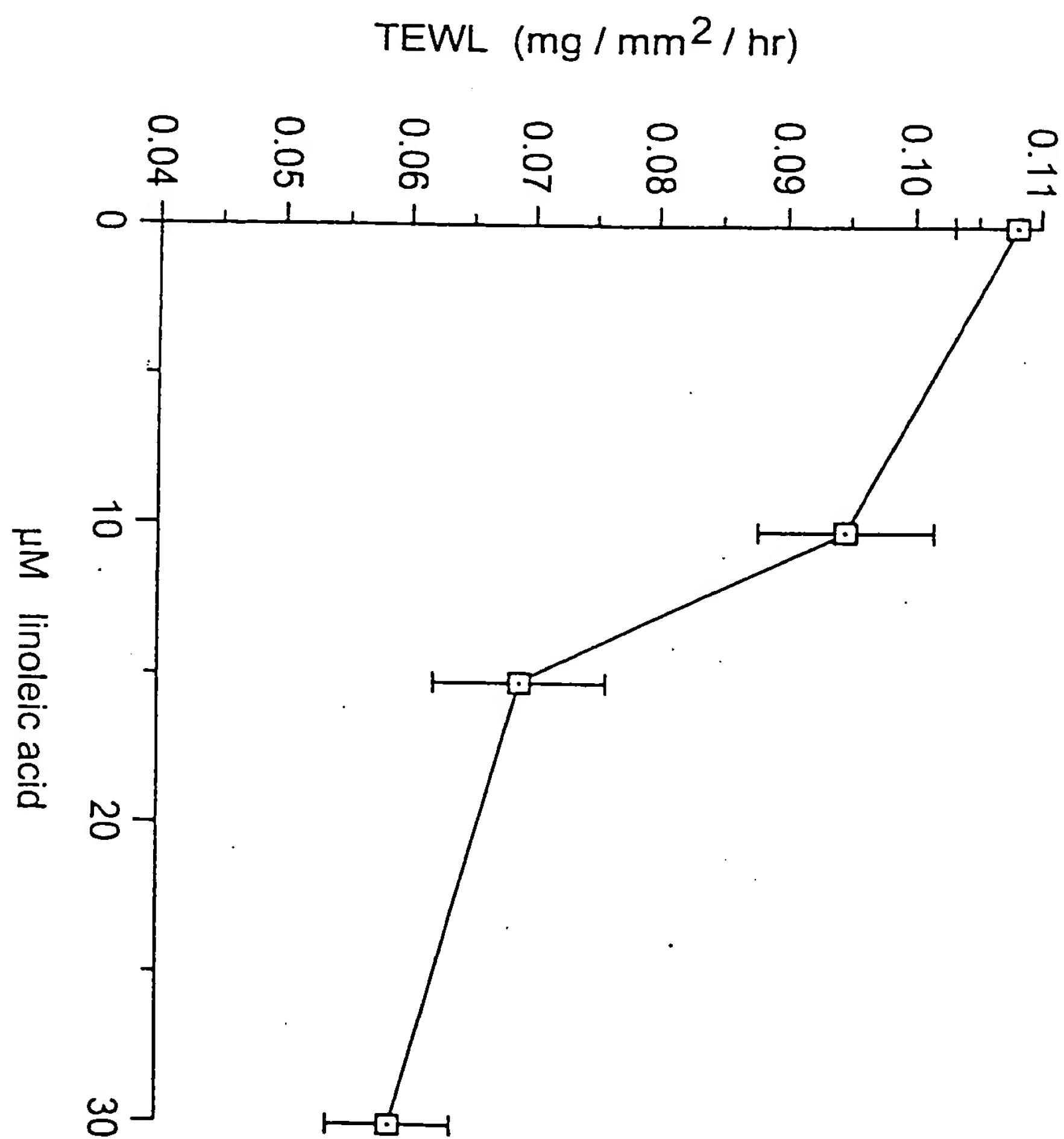


Fig. 4b

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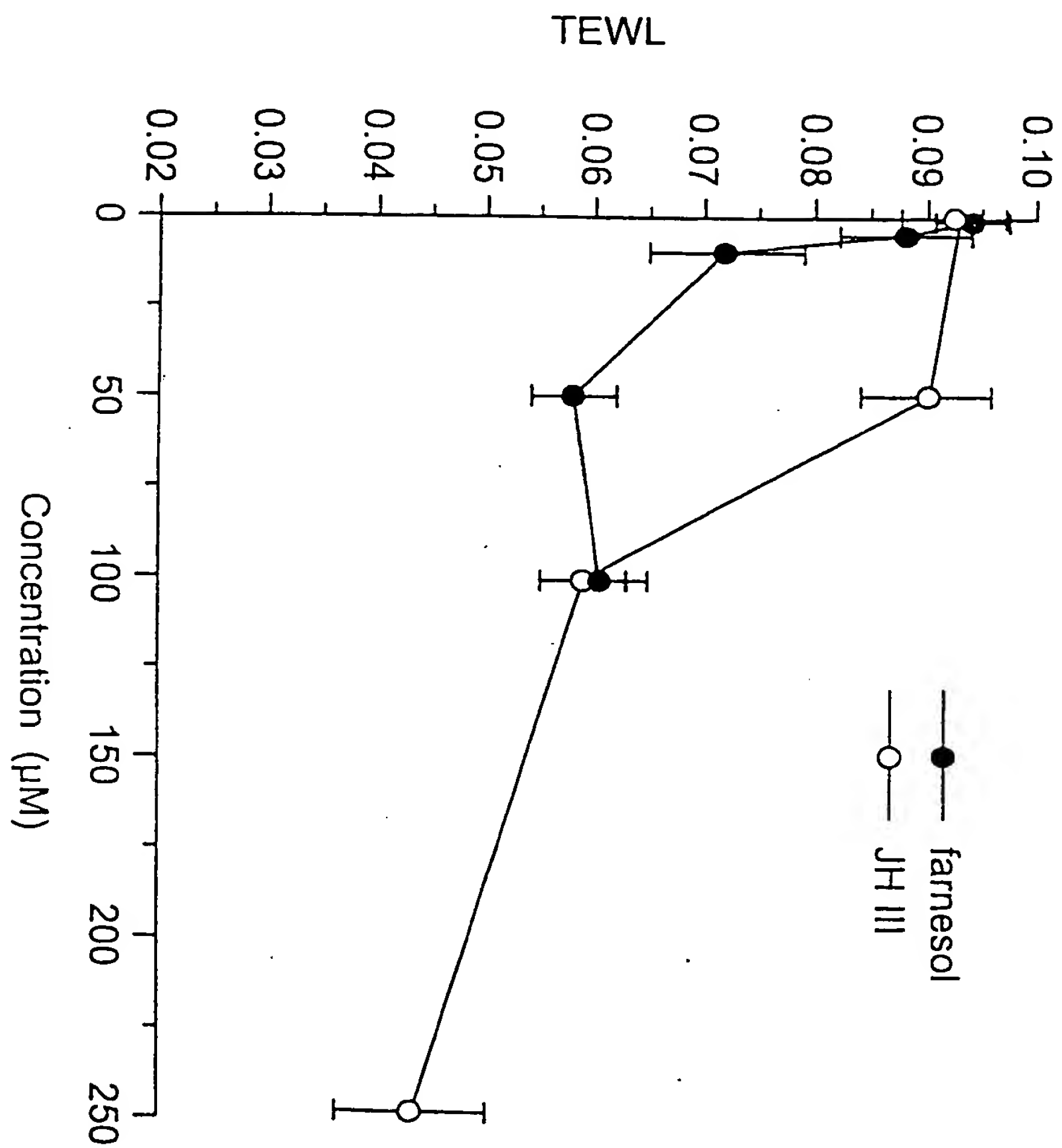


Fig. 4c

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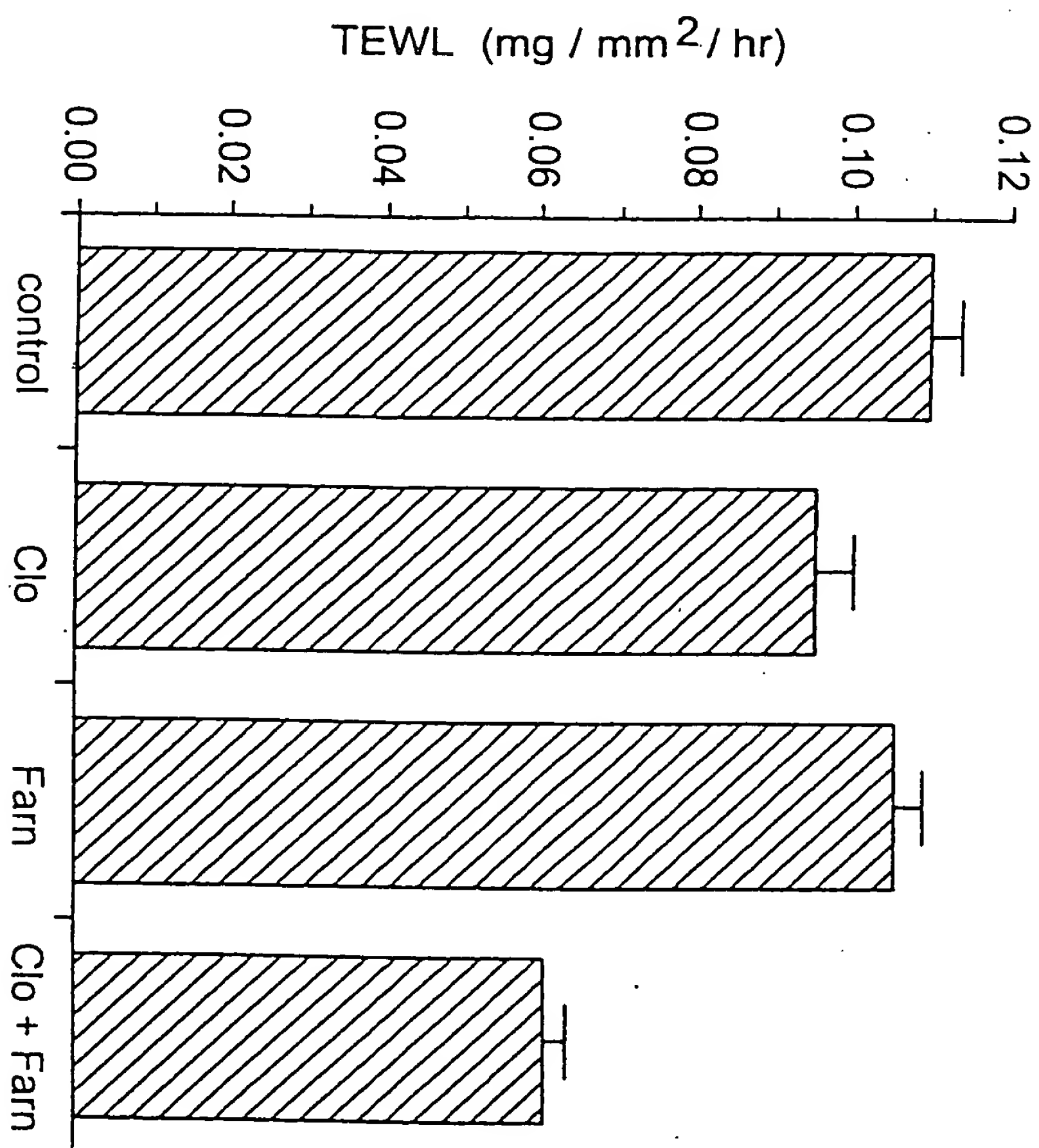


Fig. 5a

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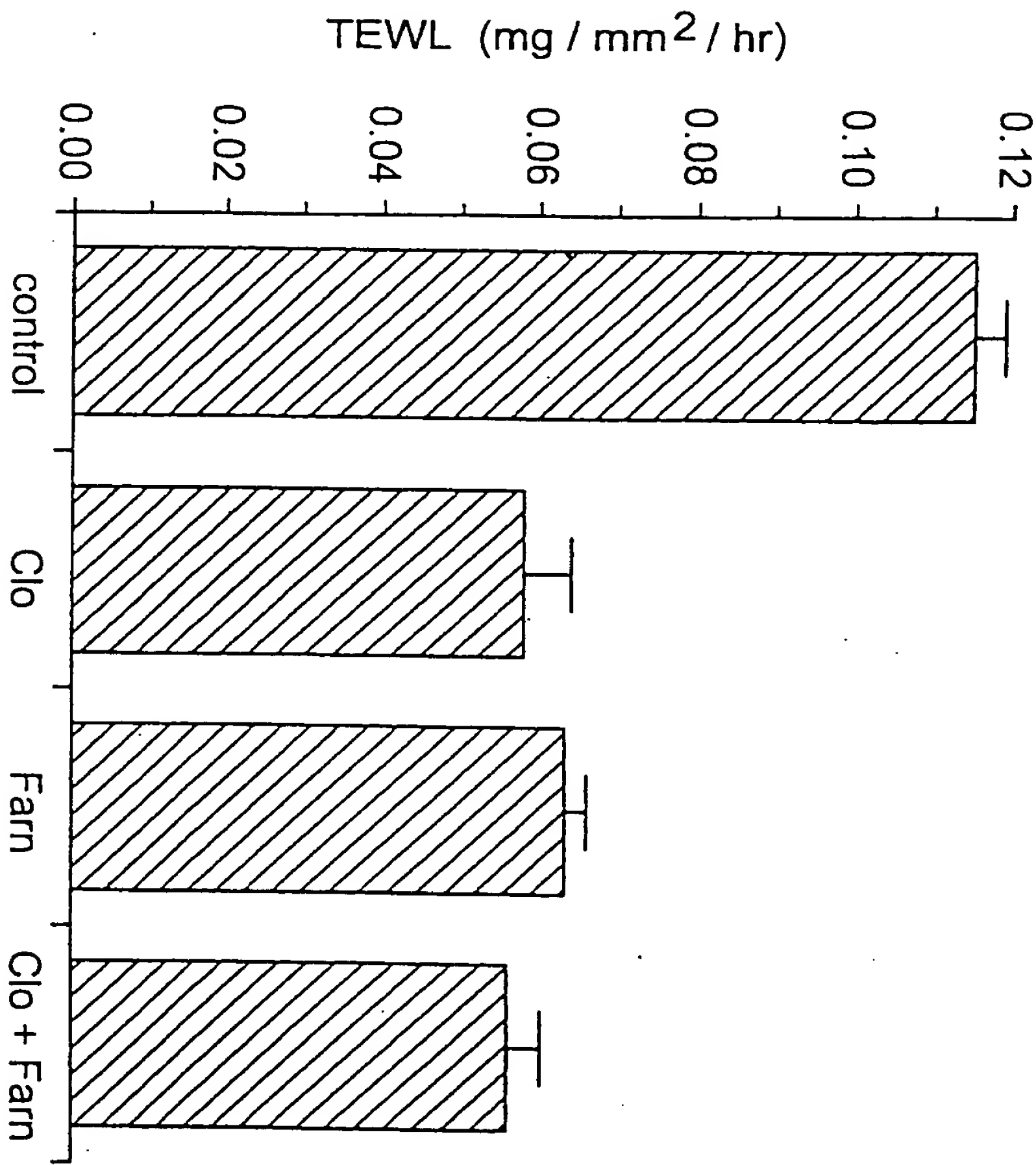
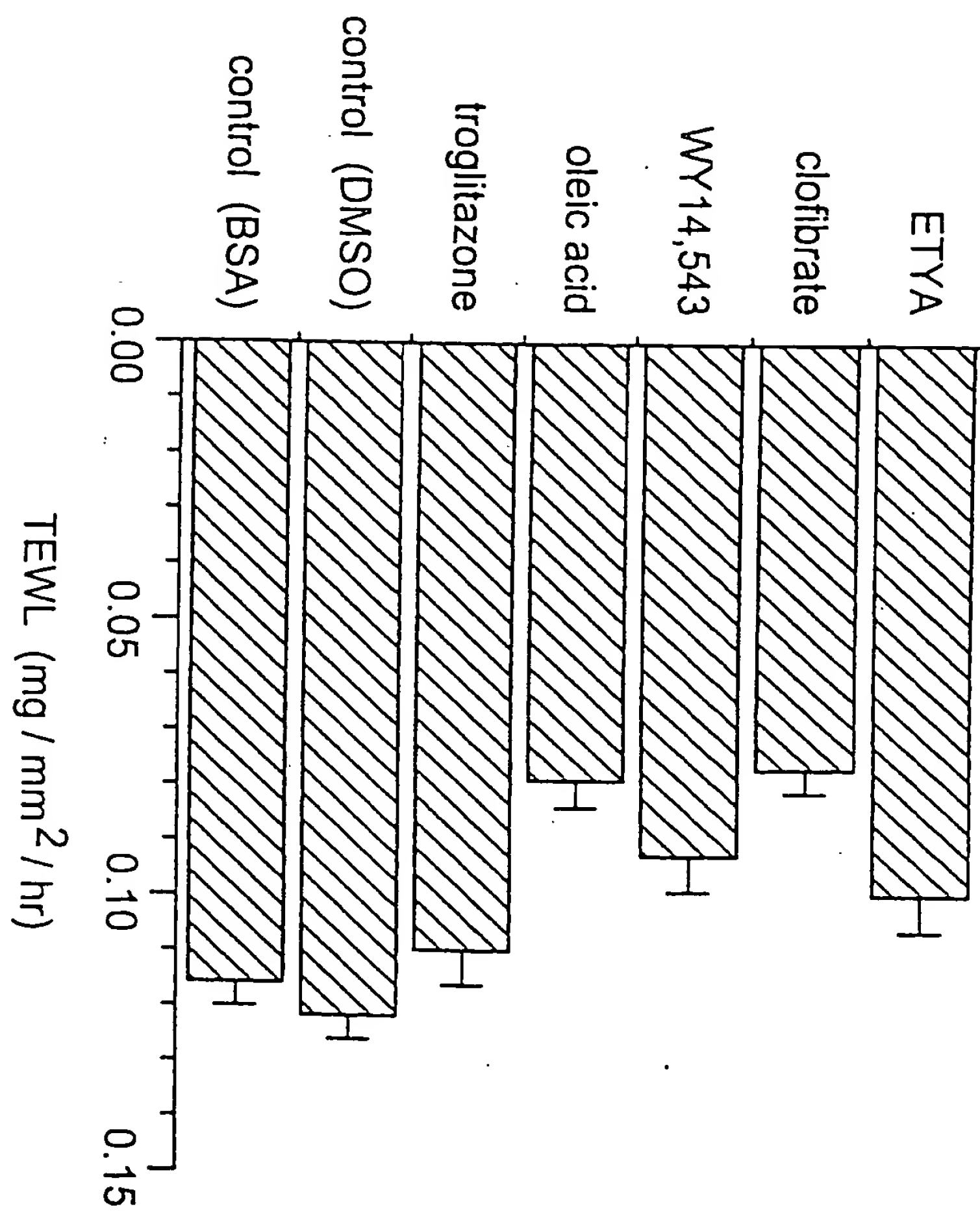
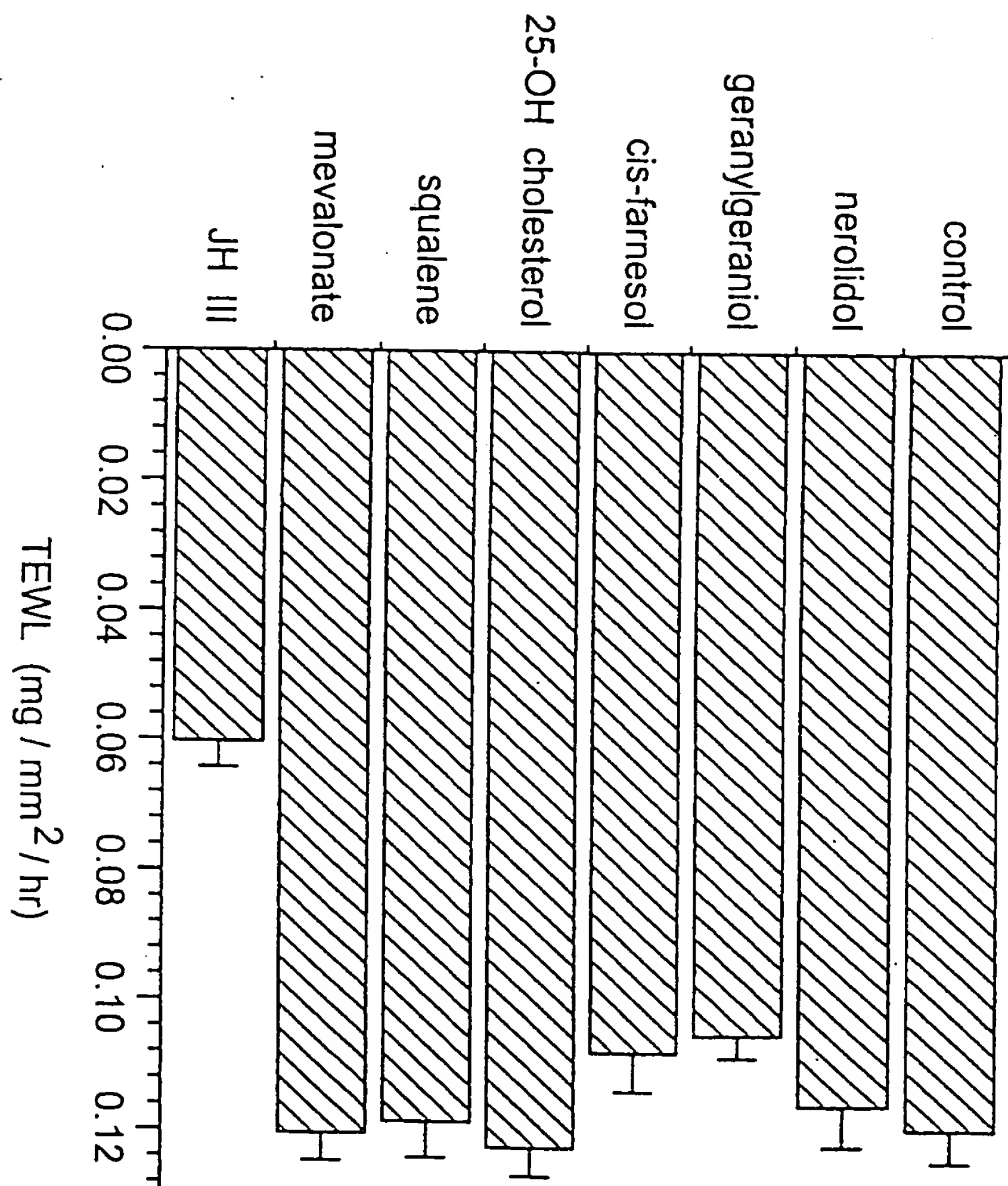


Fig. 5b

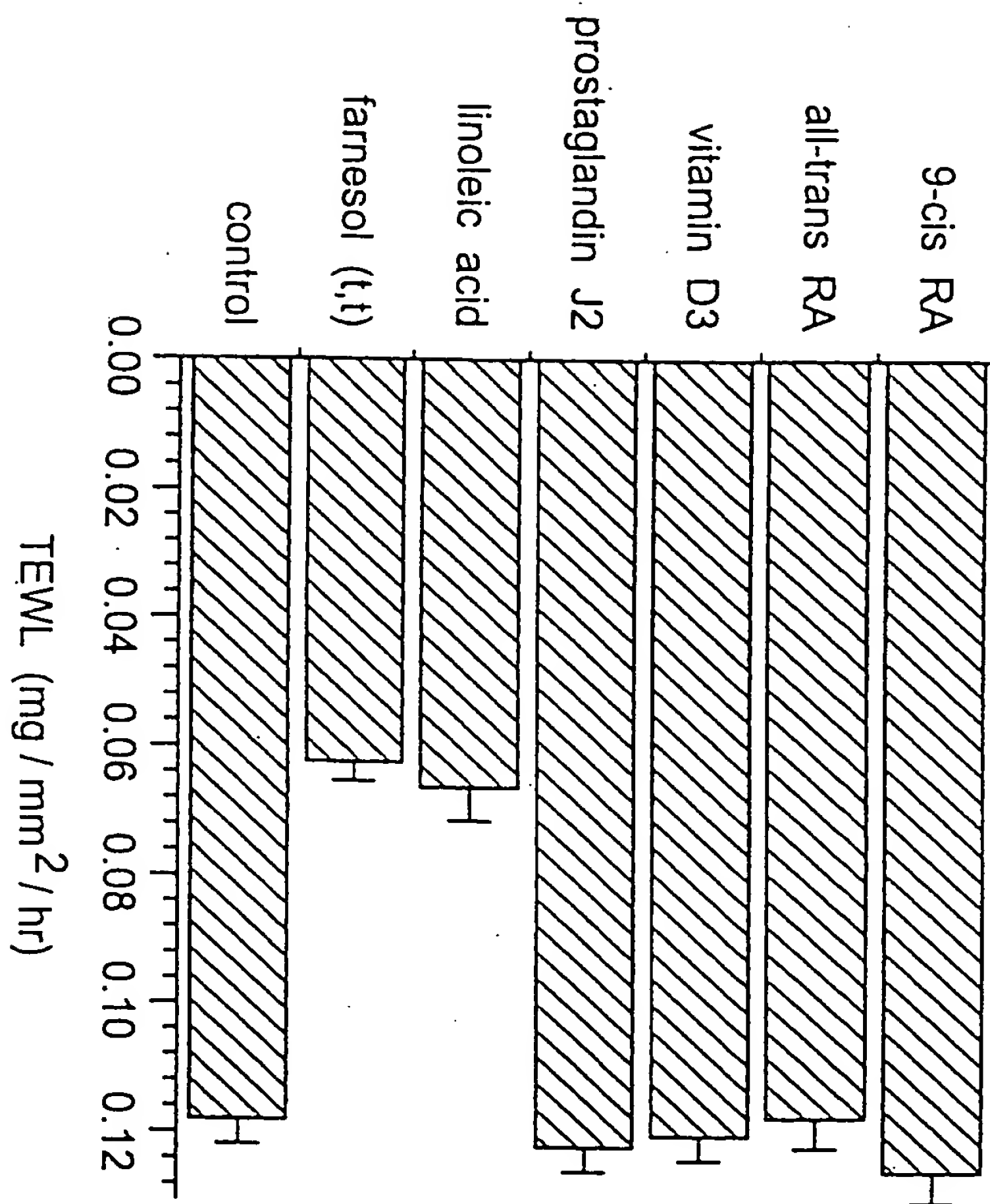
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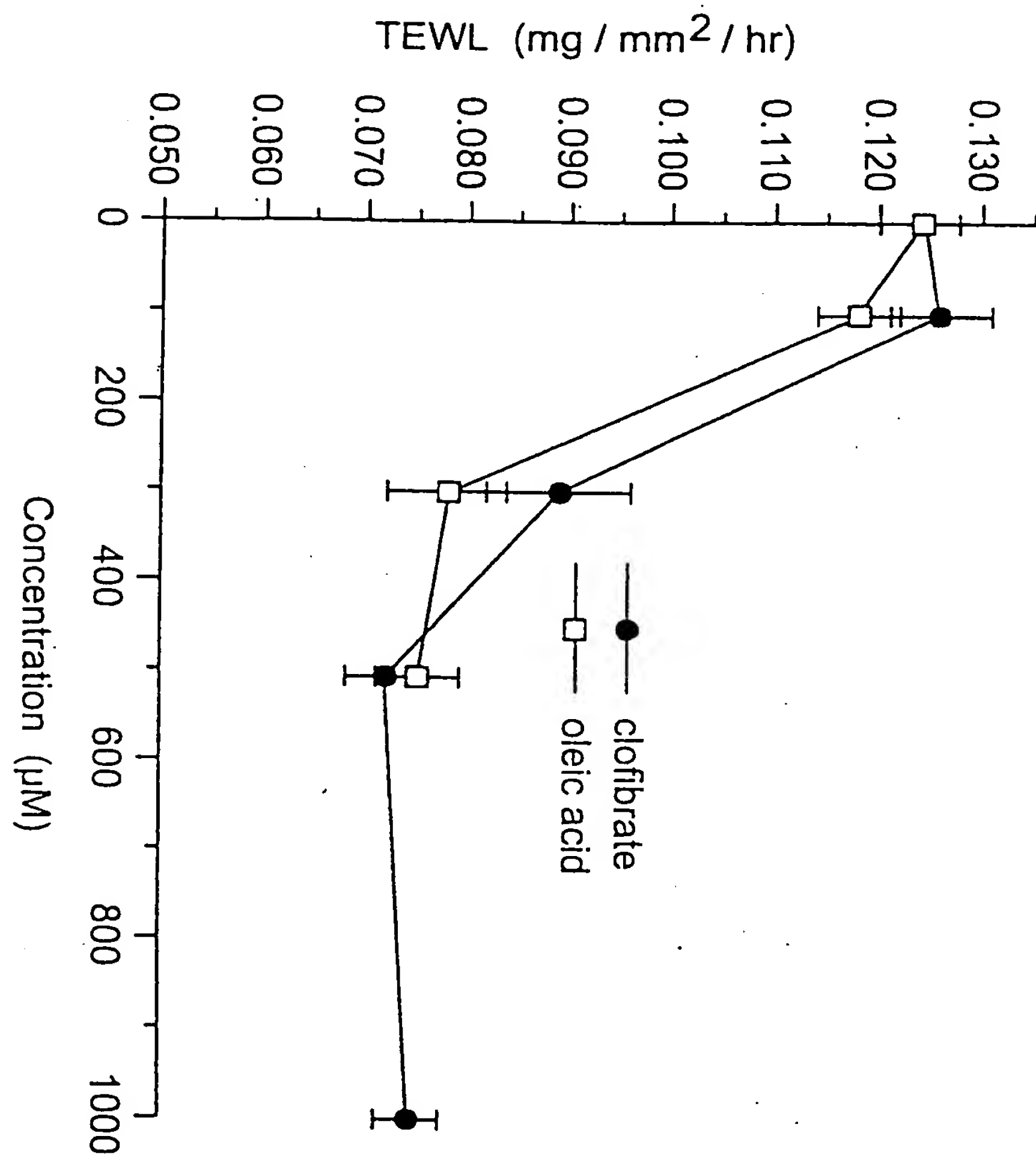
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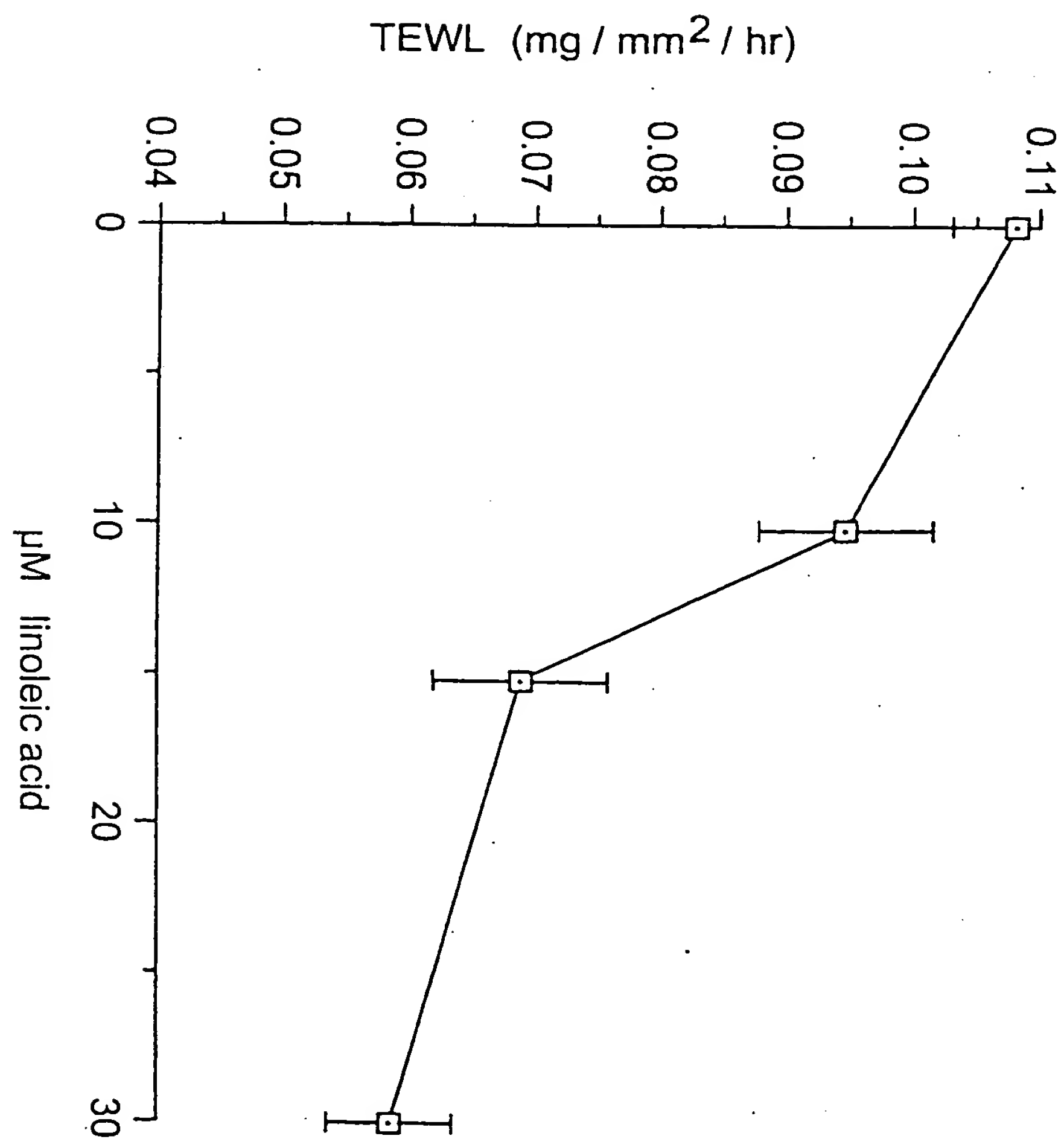
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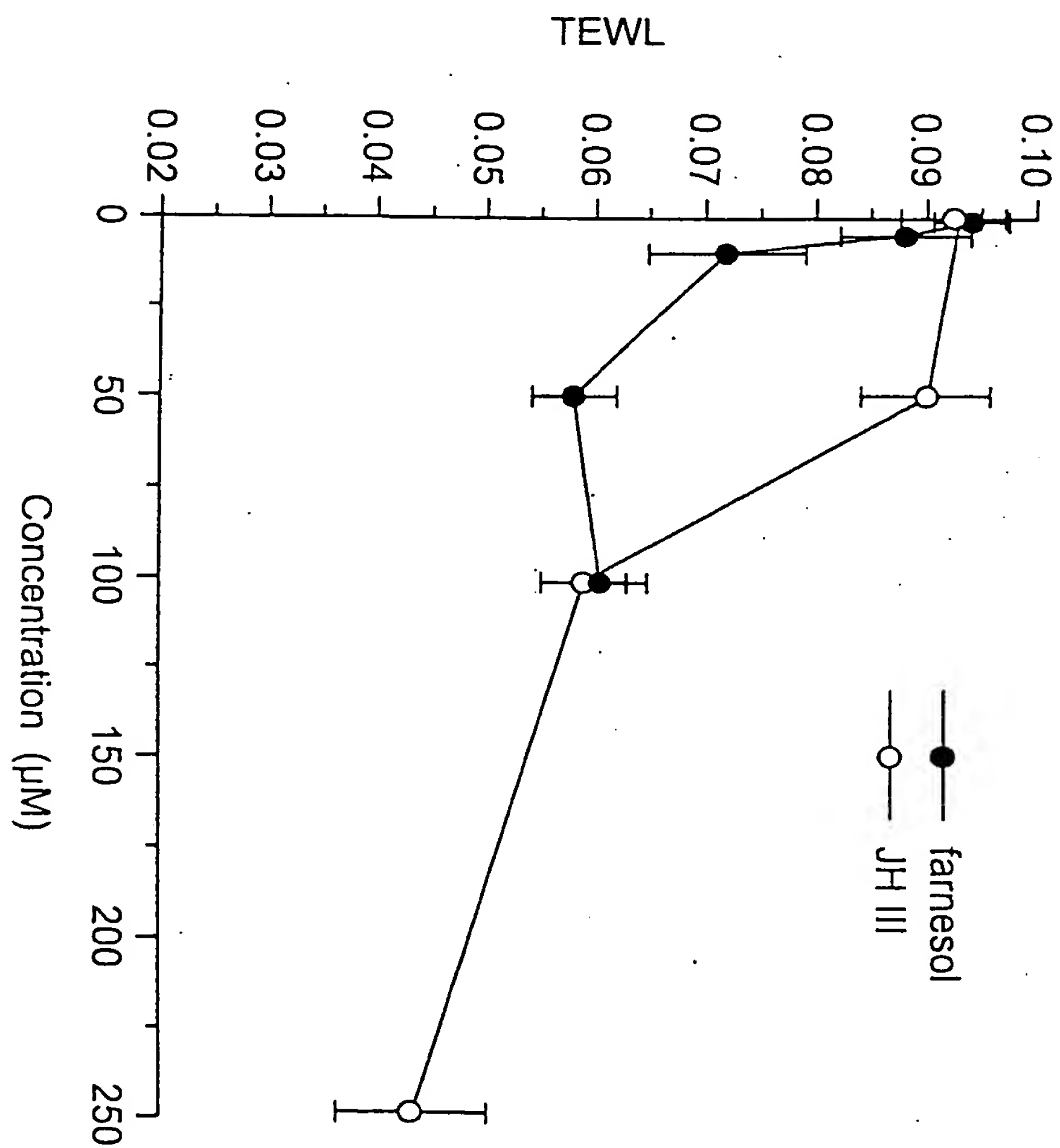
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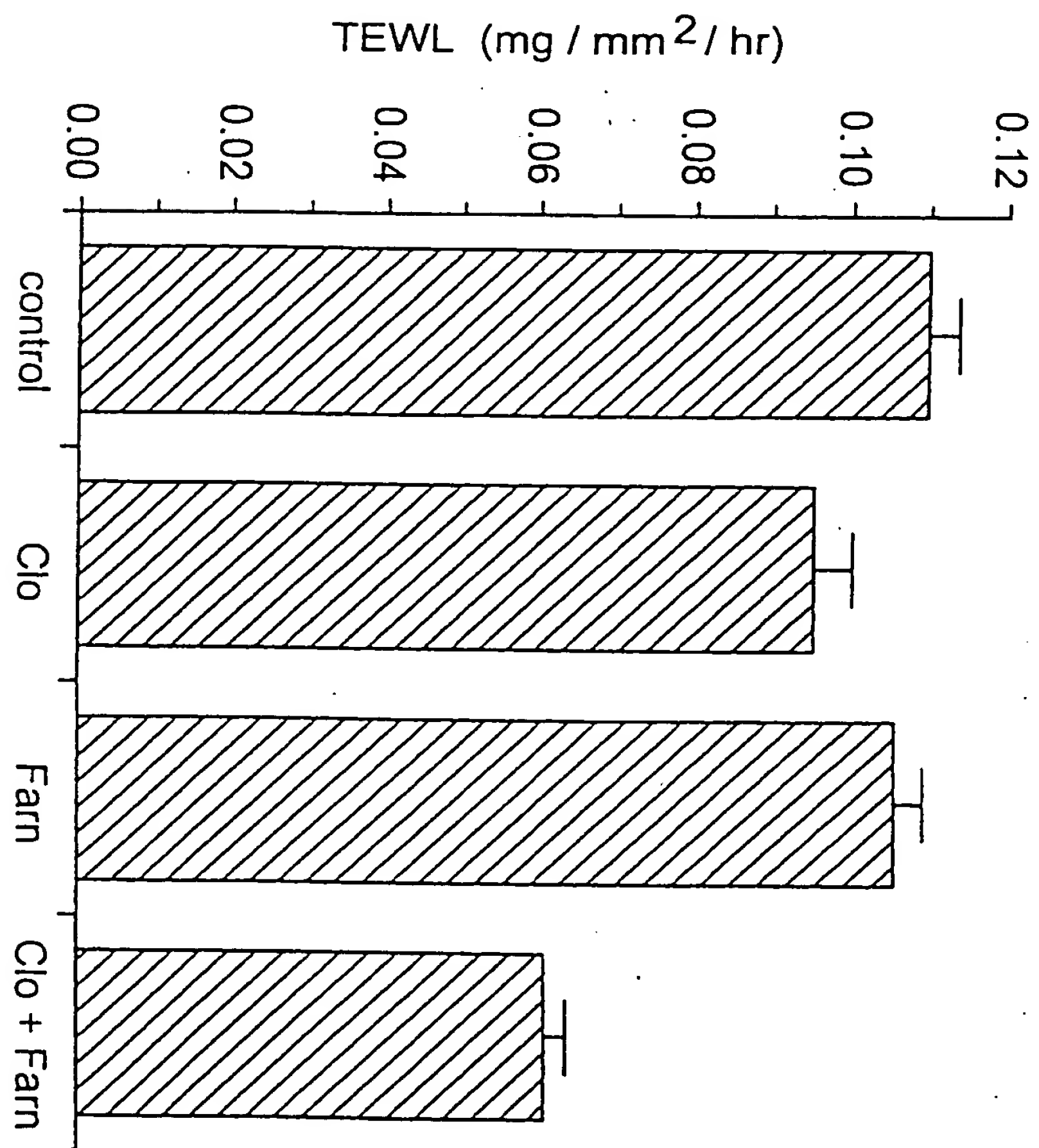
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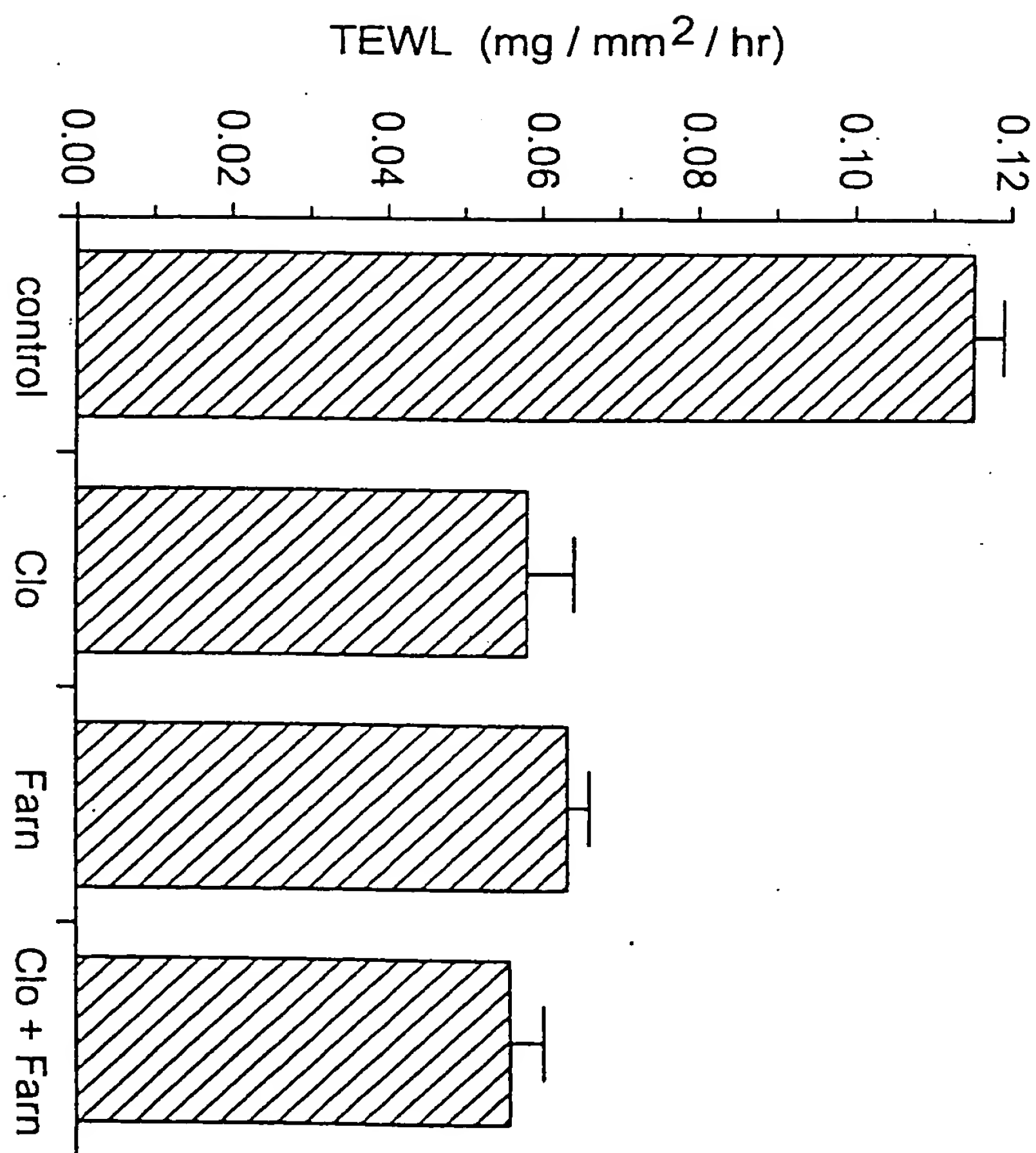
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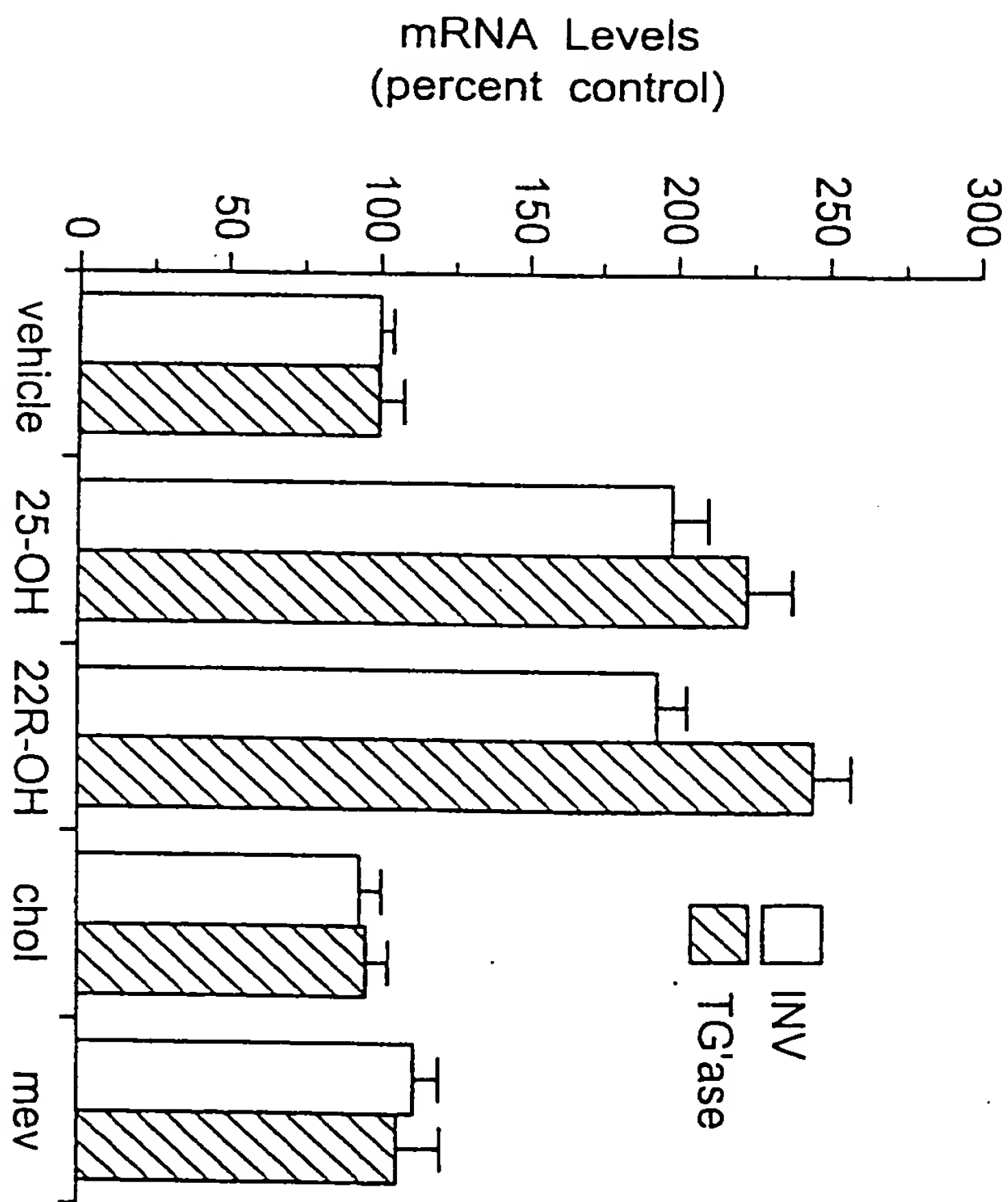


Fig. 14a

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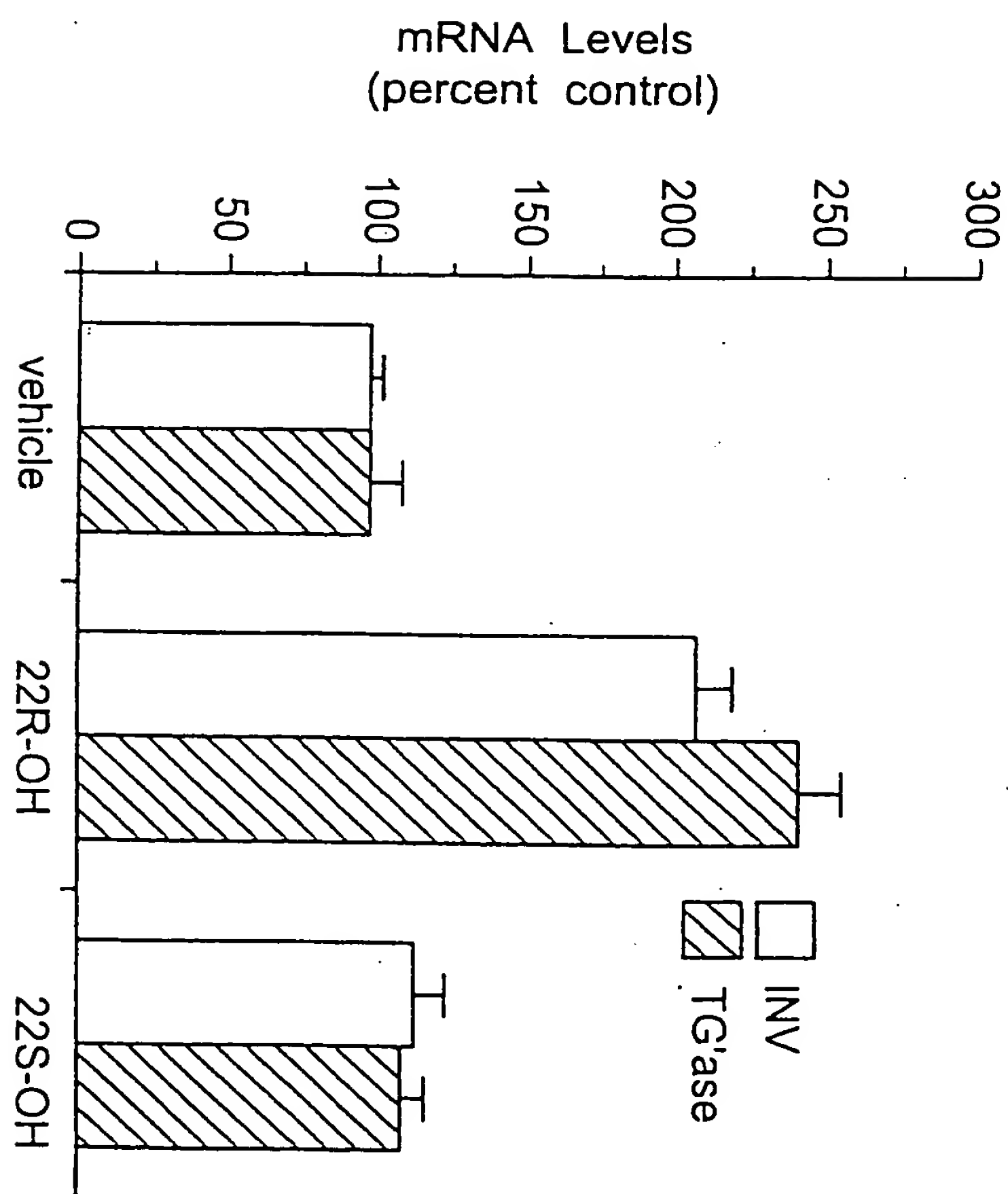


Fig. 14b

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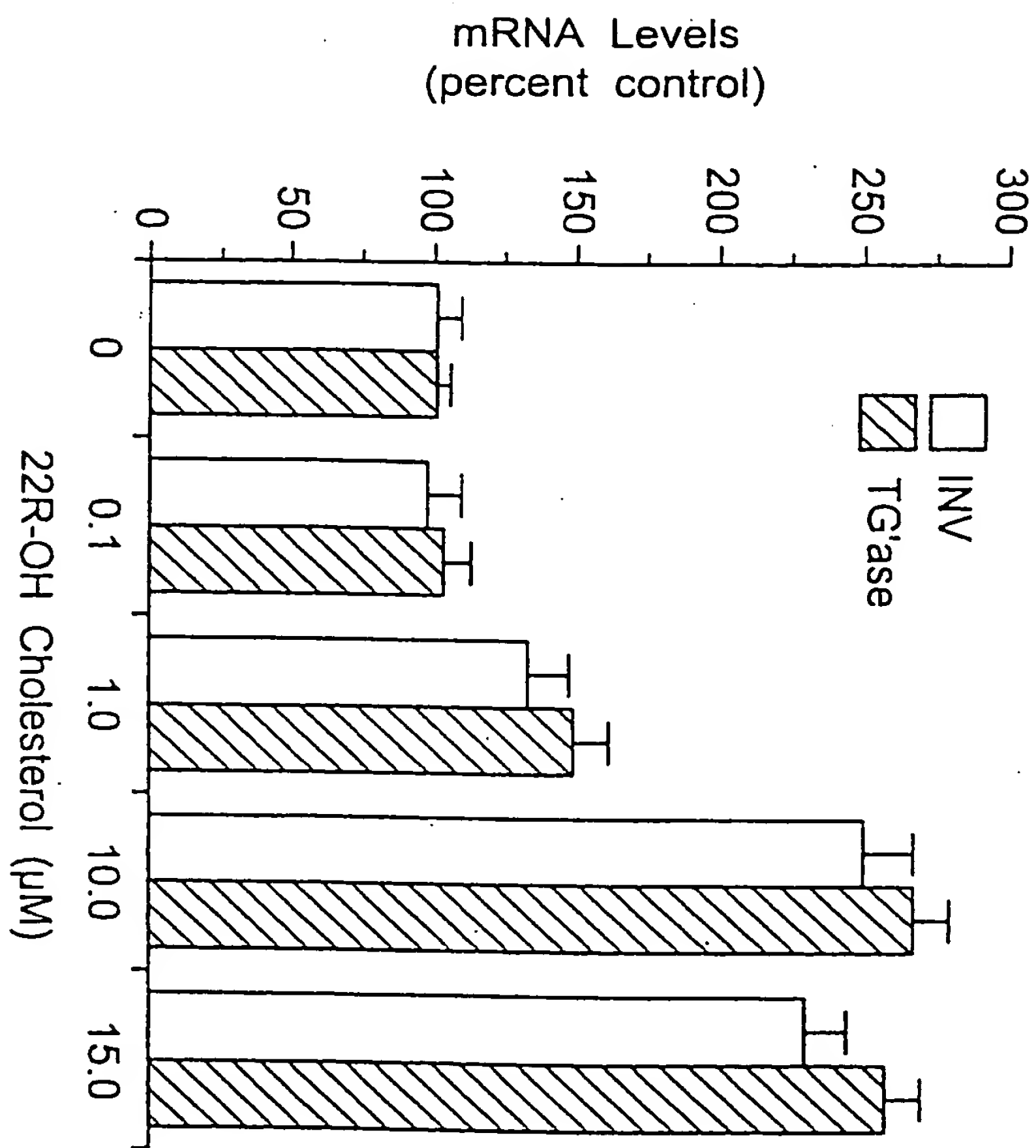


Fig. 15

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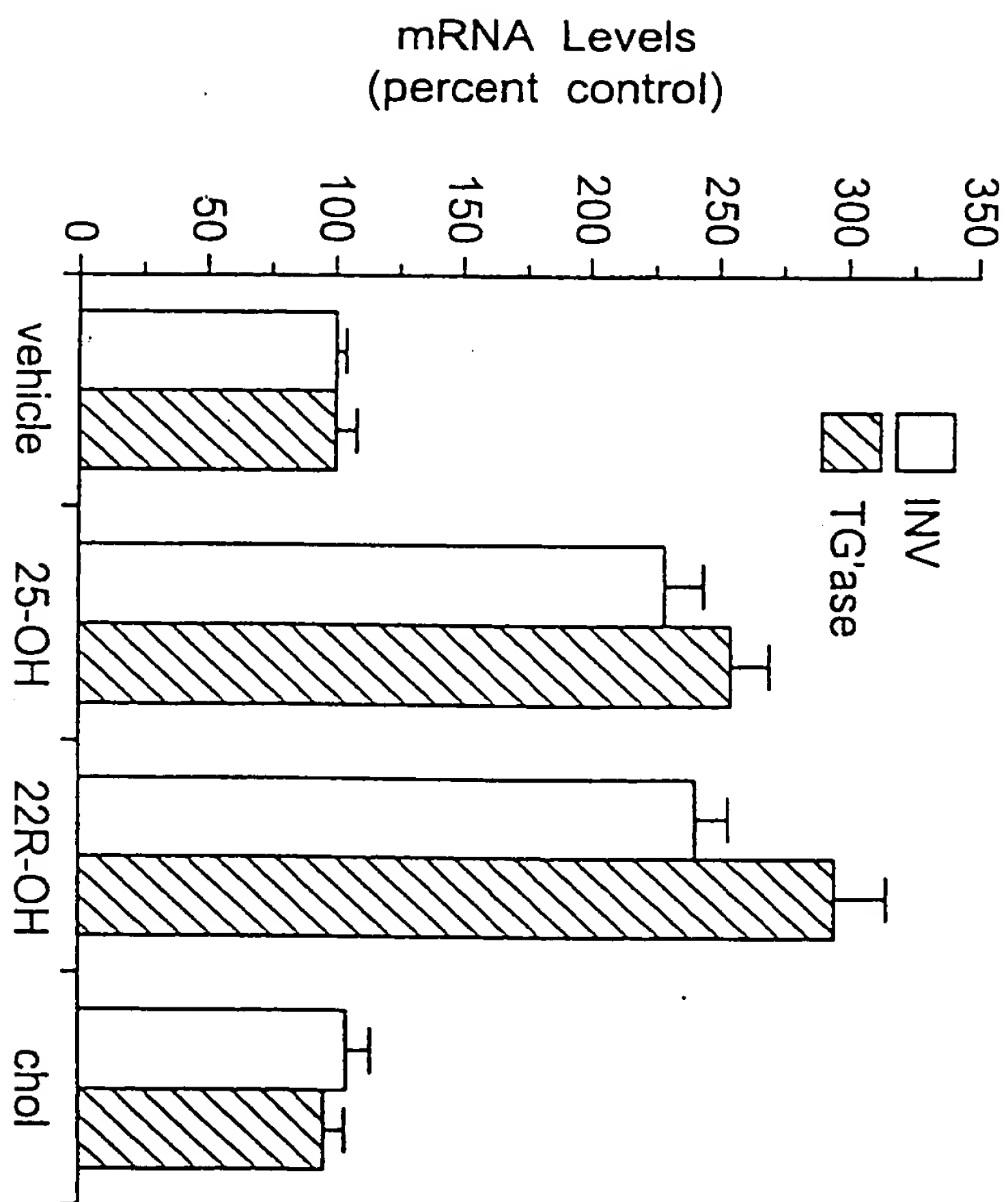


Fig. 16

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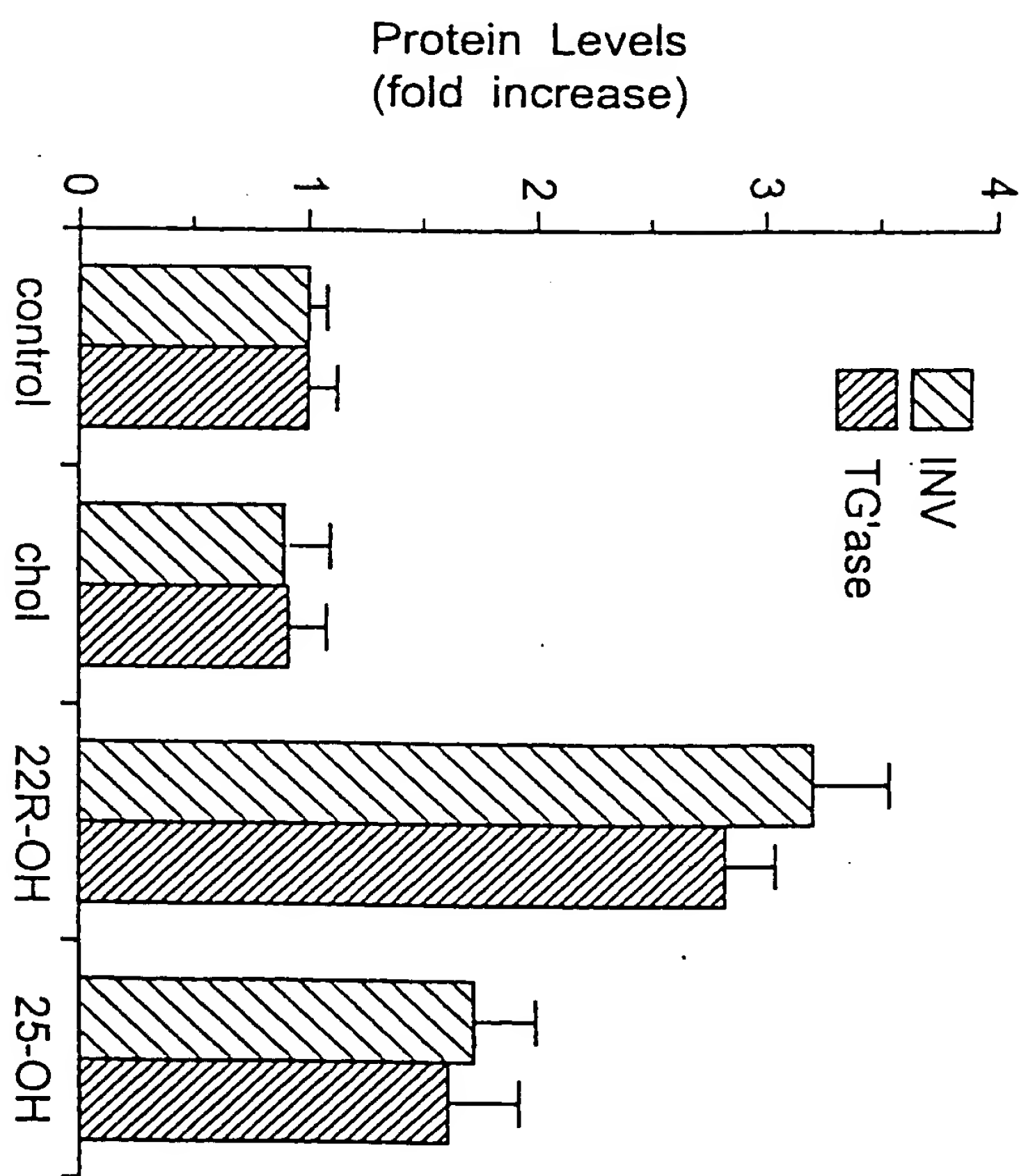


Fig. 17a

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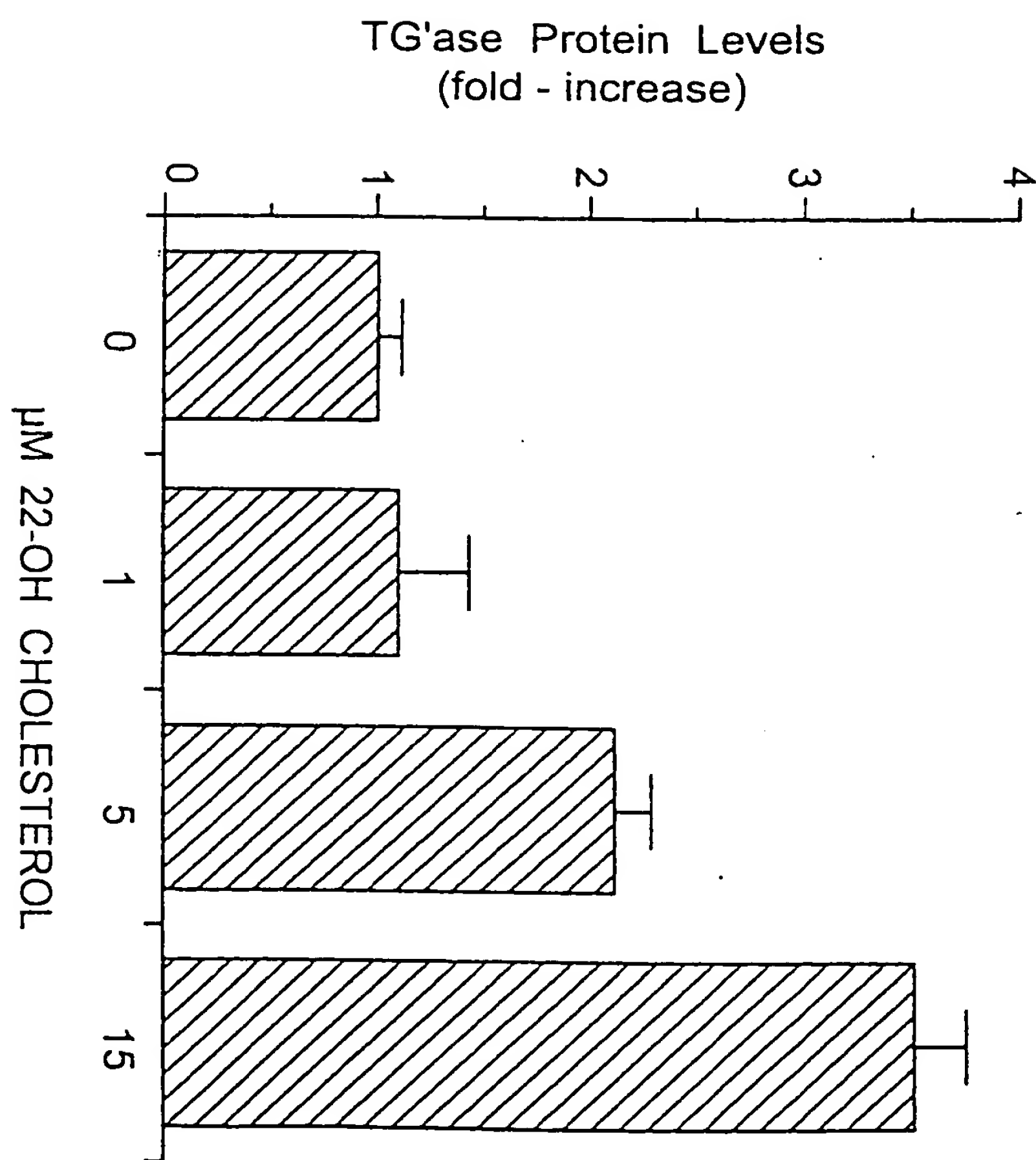


Fig. 17b

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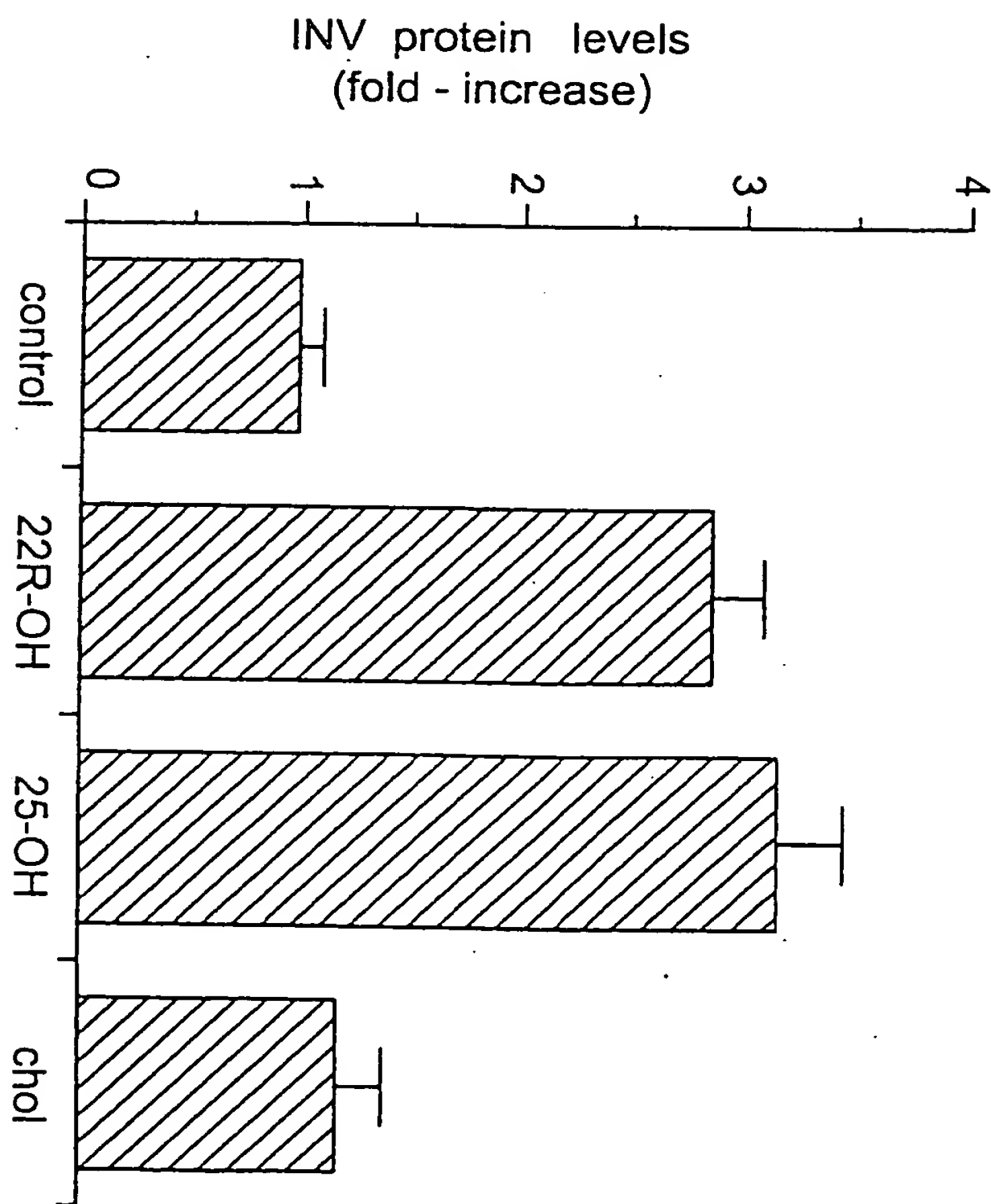


Fig. 17c

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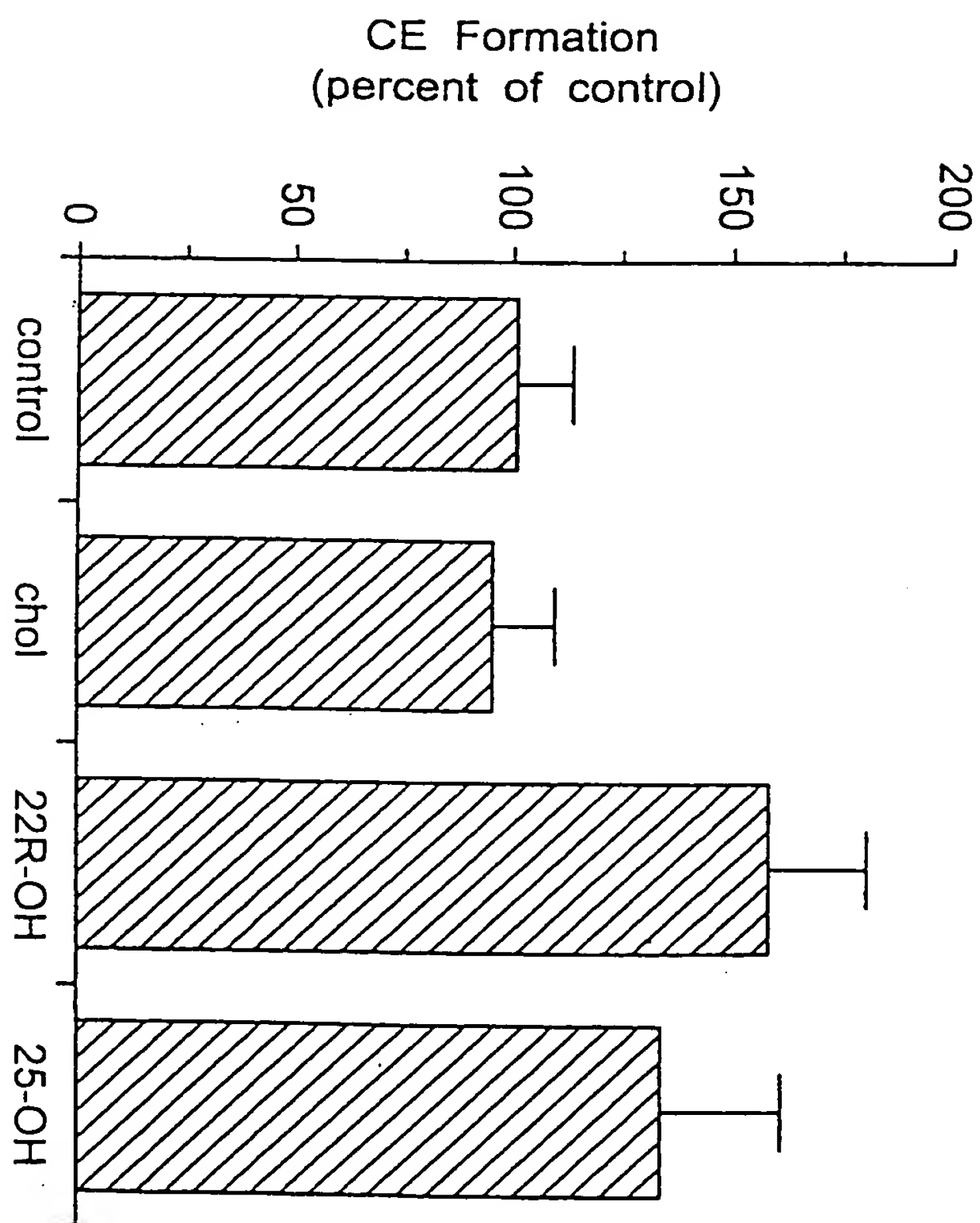


Fig. 18a

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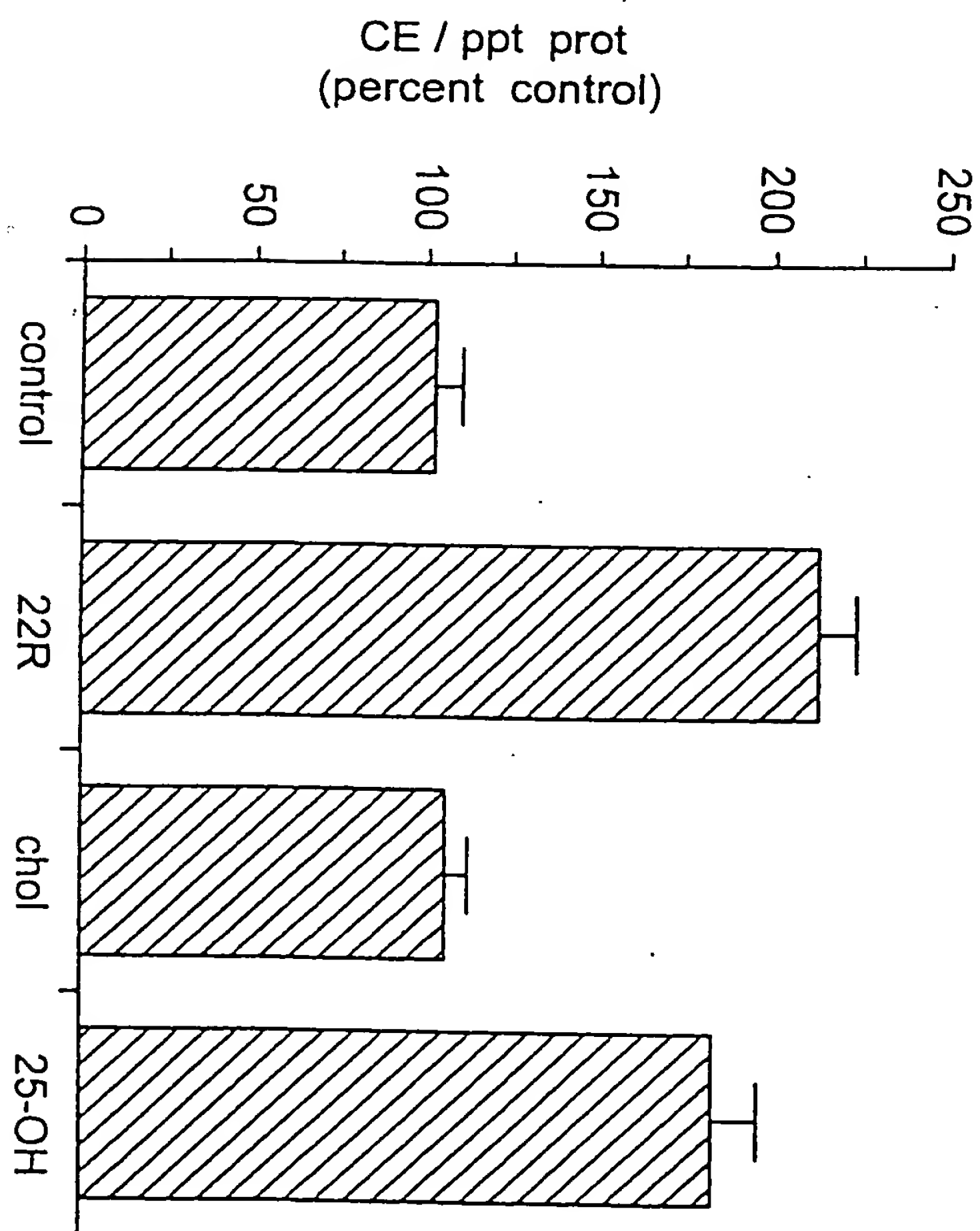


Fig. 18b

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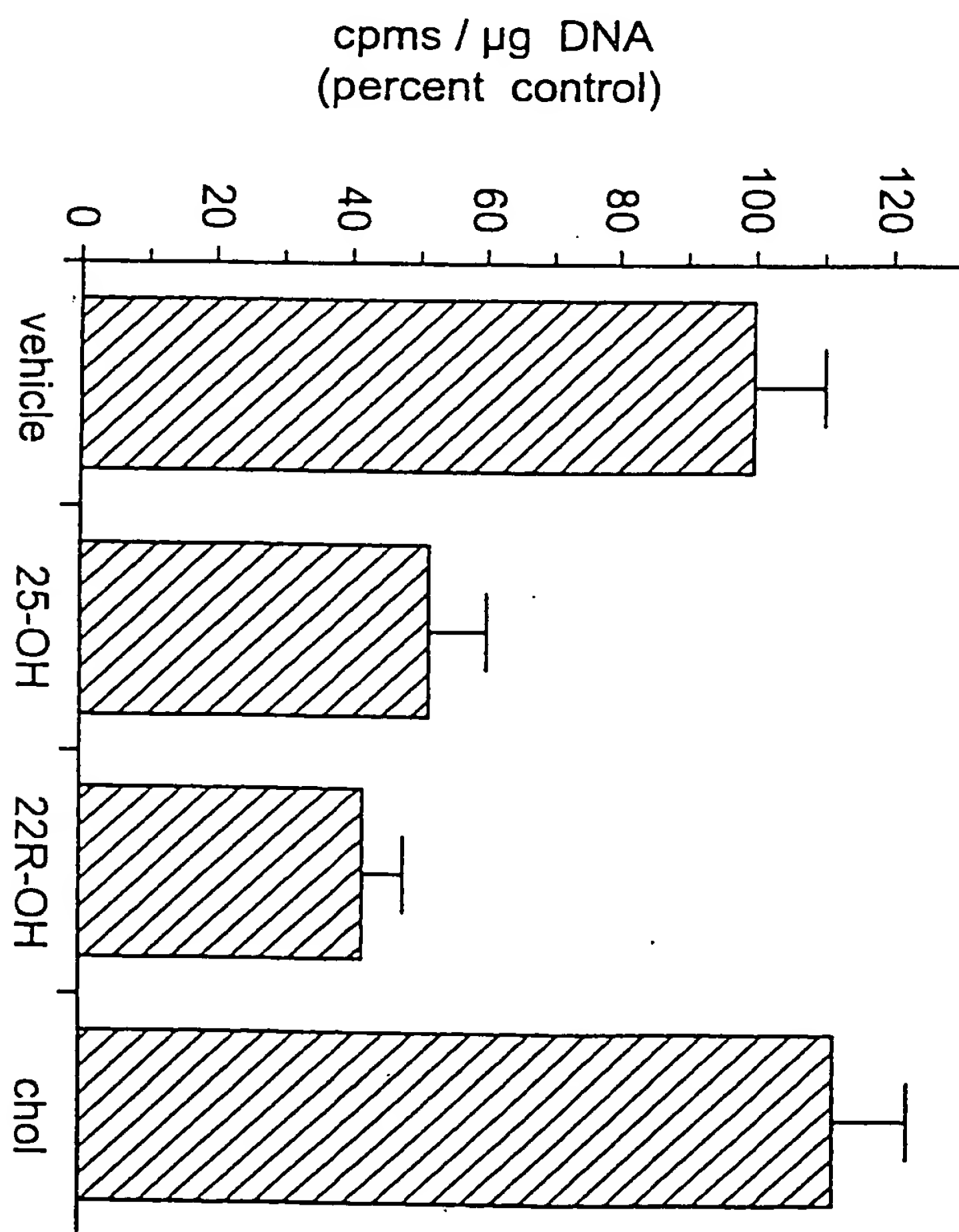


Fig. 19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/01276

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/56, 31/505, 31/335, 31/22, 31/20, 31/11, 31/45

US CL : 514/703, 739, 549, 475, 560, 272, 559, 182

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/182, 272, 475, 549, 559, 703, 739

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: Registry, HCAPLUS, USPATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0447318 B1(L'OREAL) 31 May 1995, claims 1, 19.	1-5, 11-12, 17-21, 21-28
Y	Database HCAPLUS on STN, (Columbus, OH., USA), No. 124:45666 YANG, et al., "Topical stratum corneum lipids accelerate barrier repair after tape stripping, solvent treatment and some but not all types of detergent treatment", Abstracts Br. J. Dermatol. 1995 133(5), 679-85.	1, 7-9, 11-15
X	Database HCAPLUS on STN, (Columbus OH, USA), No. 112: 208026, IMAKADO, S. et al., "Targeting expression of a dominant negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function", abstract, Genes Dev., 1995, 9(3), 317-29.	1, 7, 15-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 MARCH 1998

Date of mailing of the international search report

27 APR 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01276

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01276

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claims 1-6, 15-20 each in part, 21-26, 34-39 each in part, directed to a method of treating a condition characterized by a perturbed epidermal barrier function or disturbed differentiation or excess proliferation.

Group II, claims 1, 7-10, 15-20 each in part, 21, 27-29, 34-39 each in part, directed to a method of treating a condition characterized by a perturbed epidermal barrier function or disturbed differentiation or excess proliferation.

Group III, claims 1, 11-14, 15-20 in part, 21, 30-33, 34-39 each in part, directed to a method of treating a condition characterized by a perturbed epidermal barrier function or disturbed differentiation or excess proliferation.